

**THE ROLE OF PHOSPHORYLATED CREB IN THE PREFERENTIAL
RECRUITMENT OF IMMATURE DENTATE GRANULE NEURONS
INTO LEARNING AND MEMORY CIRCUITRY**

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ABSTRACT

Adult hippocampal neurogenesis has been linked with learning and memory in mammalian species (including humans). Recent work has demonstrated a critical period following the birth of new hippocampal neurons during which they display a competitive advantage over their neighbors and integrate preferentially into spatial memory circuitry. The current study represents a preliminary investigation into this effect, with the overarching goal of elucidating the molecular mechanism that underlies the accelerated integration of immature neurons into spatial memory circuitry. Based on its role in regulating both neurogenic processes (e.g., proliferation, maturation, and survival) and intrinsic neuronal excitability, it was hypothesized that the intracellular protein CREB might play an important role in the neurogenic basis of memory. To mark the birth of adult-generated neurons, rats were injected with the neural proliferation marker BrdU at several time-points (1, 3, 6, and 10 weeks) prior to training in the Morris Water Maze. Double-label (BrdU + pCREB) immunofluorescence in conjunction with high-powered confocal microscopy was used to visualize the extent of pCREB expression in both BrdU⁺ and BrdU⁻ cells in the granule cell layer of the hippocampus. Three week old dentate granule neurons showed greater pCREB expression in response to the MWM than did their one week old counterparts. Although qualitative analysis indicates that pCREB is more strongly expressed in 6 week old dentate granule neurons relative to neighbouring neurons, this effect failed to reach statistical significance. Based on the small sample size used in the histological portion of this study in addition to the large effect size discovered, this failure to achieve statistical significance likely represents a type II error. That is, a significant effect is present, but the current study lacked the power to detect it statistically. The potential role played by CREB in mediating the preferential recruitment of immature neurons into hippocampal-dependent learning and memory circuitry warrants further investigation.

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LIST OF ABBREVIATIONS

Arc	activity-regulated cytoskeletal associated protein, or Arg 3.1
BrdU	bromodeoxyuridine
CREB	cyclic adenosine monophosphate (cAMP) responsive element-binding protein
DNA	deoxyribonucleic acid
GABA	γ -aminobutyric acid
GCL	granule cell layer
LTD	long-term depression
LTP	long-term potentiation
MAM	methylazoxymethanol acetate
MWM	morris water maze
NeuN	neuron-specific nuclear marker
NMDA	N-Methyl-D-aspartate
pCREB	phosphorylated CREB
SGZ	subgranular zone

“In the adult centres, the nerve paths are something fixed, ended and immutable. Everything may die, nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree.”

- Santiago Ramon y Cajal.

CHAPTER 1

GENERAL INTRODUCTION

1.1. Learning and Memory

The unidirectionality of time is fundamental and has governed all happenings within the universe since its inception with the big bang some 13.7 billion years ago. At present, time's arrow is irreversible, perhaps with a single exception – the ability of animal life to remember and mentally re-experience events long past (Tulving, 2002; Dere et al. 2006). Indeed, the ability to acquire and retain information over time in memory is one of the most remarkable aspects of animal behaviour (Kandel, 2001). Not only does this ability bring with it immense adaptive value, but it also serves as the adhesive that holds our mental lives together; without memory, each moment in life would present itself as completely independent of every other moment. To explain the enigma of memory, philosophers were long reduced to analogy or metaphor (e.g., Plato provided the metaphor that memory is akin to impressions made onto a wax tablet [Radvansky, 2006]). However, the brain is no longer seen as a black box, and in recent years questions regarding the neurological nature of memory have given way to scientific investigation.

Memory is thought to involve the transient activity of individual neurons working in unison (short-term memory) alongside gene expression and de novo protein synthesis that together culminate in structural growth and large-scale modifications in synaptic morphology (long-term memory). Determining the morphological alterations that occur in the nervous system in response to learning and memory (i.e., structural plasticity) is a hot topic in contemporary neuroscience (Lamprecht and LeDoux, 2004). Structural plasticity was long thought to be restricted to alterations in neurons that were generated before birth. Indeed, the idea that the generation of new neurons was restricted to the prenatal brain was, until quite recently, a central tenet in neuroscience. However, over the past two decades, the evidence in favor of the existence of adult neurogenesis has become overwhelming. Adult neurogenesis represents an unprecedented form of structural plasticity in which entirely new information processing units (i.e., neurons) are added to the system. Today, adult neurogenesis is one of the most exciting and

heavily studied areas in the neurosciences, particularly in terms of the functional impact these neurons have on learning and memory (Deng et al., 2010).

1.2. Neurogenesis: A Historical Perspective

Some of the first evidence challenging the prenatal exclusivity of neurogenesis was in 1912 when dividing cells were found in the lateral ventricles of 120 day old mature rats (Allen, 1912). However, largely because neuron-specific markers did not yet exist, it was impossible to determine if the product of these cell divisions was neuronal. Furthermore, it was possible that the deoxyribonucleic acid (DNA) in the cells was not being replicated but rather was undergoing repair, and that this repair was responsible for the labeling (see Balu & Lucki, 2009, for a brief historical overview). As such, this datum was largely ignored by the neuroscience community. About 50 years later, a postdoctoral fellow named Joseph Altman published a series of high-impact papers in which he provided evidence for the birth of new neurons in the adult olfactory bulb and hippocampal dentate gyrus, a region now heavily implicated in memory (Altman 1962, 1963 1969; Altman and Das, 1965ab). Once again, however, these results were largely disregarded by the neuroscience community, likely for the following reasons (as reviewed by Gross, 2000). First, the techniques available at the time were not sufficient for definitively determining that the adult generated cells were neurons rather than glia. Second, the results were considered unprecedented and thus unlikely, particularly in terms of the long migratory path taken by olfactory system cells (i.e., from the lateral ventricles to the olfactory bulbs). Lastly, Altman was an unknown figure at the time whose results would have overturned a universally held dogma in the neurosciences. Not surprisingly, there was intellectual resistance to this previously unrecognized and remarkable form of plasticity in the adult brain. Indeed, even as late as 1970, one authoritative textbook on developmental neurobiology stated that “...there is no convincing evidence of neuron production in the brains of adult mammals” (Jacobson, 1970, as cited in Gross, 2000).

Starting about fifteen years later, Michael Kaplan followed up on the work of Altman using electron microscopy. With this technology, he was able to provide ultra-structural evidence (in the form of axons, dendrites and synapses) that the dividing cells seen in the olfactory bulb and dentate gyrus were neuronal in lineage (Kaplan, 1981; Kaplan, 1983; Kaplan, 1985ab; Kaplan and Hinds, 1977). Kaplan’s work was also ignored, likely for many of the same reasons

underlying the dismissal of Altman's results. Especially damning was the fact that Pasko Rakic, an influential and leading researcher in primate brain development at the time was unable to replicate Kaplan's results in Rhesus monkey, finding not "a single heavily labeled cell with the morphological characteristics of a neuron in any brain of any adult" (Rakic, 1985). In fact, Rakic went on to argue that this result made sense because "a stable population of neurons in primates, including humans, may be important for the continuity of learning and memory over a lifetime" (Rakic, 1985). As shall be discussed, Rakic was quite wrong on this point, as neurogenesis is now thought to be adaptive and contribute significantly to learning and memory ability in mammals. Unfortunately, Kaplan found the non-acceptance of his work too much to bear, and left biomedical research for a career in medicine (see Kaplan, 2000, for his account of the neurogenic 'dogma' that he helped overturn).

There are three factors that seem to have led to the eventual acceptance of adult neurogenesis as a genuine, significant, and interesting phenomena within the neuroscientific community (as outlined by Gross, 2000): First, a highly respected scientist by the name of Fernando Nottebohm unambiguously demonstrated neurogenesis in the brains of adult birds in an elegant series of experiments (Nottebohm, 1985; Nottebohm, 1996; Goldman and Nottebohm, 1983; Burd and Nottebohm, 1985; Paton and Nottebohm, 1984; Barnea and Nottebohm, 1994; Kirn and Nottebohm, 1993). Second, the introduction of neuron-specific immunohistochemical markers in conjunction with the development of Bromodeoxyuridine (BrdU; detailed below) provided strong evidence, especially when combined with the structural studies, that the dividing cells were neuronal in lineage. However, it was still possible that neurogenesis was an evolutionary vestige. That is, it was possible that new neurons were generated in the adult brain, but that these neurons served no significant or interesting physiological function. Thus, the third and final breakthrough that led to the scientific acceptance of adult neurogenesis as an important phenomena was the discovery that levels of hippocampal neurogenesis were readily regulated by psychological variables such as stress (Gould and Tanapat, 1999), environmental enrichment (Kempermann et al., 1997), and learning (Gould et al., 1999). Importantly, factors that are typically conceptualized as maladaptive (e.g. chronic stress) resulted in decreased levels of neurogenesis, whereas factors generally viewed as adaptive (e.g., environmental enrichment or learning) resulted in increased levels of neurogenesis. These results not only helped solidify the existence of hippocampal neurogenesis in adult mammals, but they also suggested that the birth

and subsequent integration of these new neurons might somehow contribute to hippocampal plasticity in a functionally relevant way.

1.3. The Neurogenic Brain

Now, approximately 100 years after Allen (1912) discovered mitotically dividing cells in the brains of adult rats and approximately 50 years after Altman (1962) provided convincing evidence that these dividing cells were neurons, the existence of neurogenesis in the adult mammalian brain is a universally accepted phenomenon. There are two regions in the brain that are neurogenic under physiologically normal conditions, one being the olfactory system and the other being the hippocampus. In the olfactory system, precursor cells are found in the subventricular zone of the walls of the lateral ventricles where they eventually migrate into the olfactory bulb and differentiate into either granule or inhibitory interneurons (Balu and Lucki, 2009; Lois and Alvarez-Buylla, 1993; Doetsch et al. 1999; see Gould, 1999, for evidence of cortical neurogenesis in the adult primate brain under basal conditions). In the hippocampus, precursor cells are located in the subgranular zone (SGZ) of the dentate gyrus where they eventually migrate into the granule cell layer (GCL) and mature into fully functioning neurons. Due in large part to its critical role in cognition and the regulation of emotion (e.g., its involvement in the hypothalamic-pituitary-adrenal axis), the birth of new neurons in the hippocampus has received more interest than that in the olfactory system. Consistent with this, the current thesis will focus on the maturation and integration of dentate gyrus granule neurons.

Adult stem cells are characterized by their ability to continually proliferate (i.e., undergo mitosis) and their multipotency (i.e., their ability to differentiate into at least two different cell types). Precursor or progenitor cells are cells that have matured to the point where they are no longer multipotent and are instead restricted to a single cell type (e.g., neuronal or glial). In the dentate gyrus of the hippocampus, precursor cells reside in the SGZ, a 2-3 cell-width thick layer in between the GCL and hilus. Once a new neuron has been generated by a progenitor cell, it migrates into the GCL and usually becomes an excitatory granule neuron. However, there is also evidence that a small but significant proportion of these cells also become inhibitory GABAergic interneurons (Liu et al., 2003).

Neurogenesis itself is a complex multi-step process involving the following steps: (1) proliferation (i.e., generation of a new cell), (2) differentiation (e.g., neuronal vs glial), and (3)

migration (e.g., into the GCL). The process of neurogenesis is complete once (4) the newly generated neuron integrates into surrounding neural circuitry and develops properties that resemble those of mature neurons (for an insightful review, see Abrous et al., 2005). In rats, 4000-9000 new granule cells are added to the hippocampus each day, a figure that extrapolates to between 90000-270000 new cells each month (depending on the age of the rat, as rates of neurogenesis diminish with age; Cameron and McKay, 2001; Rao and Shetty, 2004). Approximately 75% of these cells generated in the hippocampus differentiate into neurons (Rao and Shetty, 2004). The vast majority of the adult-generated cells in the GCL differentiate into neurons, whereas those located in the hilus have a higher probability of differentiating into glial cells (Cameron et al., 1993; Gage et al., 1995). A large majority of the cells that are generated in the adult brain will experience apoptotic cell death prior to being able to integrate into the surrounding circuit. Indeed, approximately 60% of the cells that do not successfully differentiate within 1 week of their generation will die (Dayer et al., 2003). Furthermore, of those cells that do survive this critical period, approximately 50% will undergo programmed cell death within the next 2 weeks (Dayer et al., 2003). Thus, approximately 70% of cells that begin differentiation will not survive past 3 weeks. However, those that survive this initial 3 week period will continue to survive for at least an additional 5 months and potentially permanently (Dayer et al., 2003). It has been estimated elsewhere that by the end of the rats life, the existing adult-born population of granule neurons reaches upwards of 1,000,000, amounting to about 40% of the total population of dentate granule neurons (Snyder and Cameron, 2011). Clearly, a population of this magnitude should have a significant impact on hippocampal functionality – a point that will be expanded upon in a later section. Because each granule neuron contacts around 10-15 CA3 pyramidal neurons (Acsady et al. 1998), each of the approximately 500,000 CA3 pyramidal cells in a mature animal could potentially have a direct connection from an adult generated granule neuron (Snyder and Cameron, 2011). Additionally, the connection between granule neurons and CA3 pyramidal neurons is so powerful that a single granule neuron is able to trigger activity in its CA3 targets (Henze et al., 2002). This fact, in conjunction with the reciprocal connections amongst CA3 pyramidal neurons themselves (Snyder and Cameron, 2011), suggests that even a small number of adult-generated granule neurons can have a disproportionately large impact in terms of hippocampal functionality.

At this point, it is important to note that rates of neurogenesis have been reported as significantly lower in primate hippocampus (i.e., relative to rodent hippocampus). For example, Kornak and Rakic (1999) have reported that the rate of neurogenesis is approximately 10X lower in the macaque monkey, compared to rodents. Furthermore, granule cell maturation in the dentate gyrus of non-human primates (once again using macaque monkeys as subjects) takes upwards of 6X longer than cell maturation times in rodents (Kohler et al., 2011). In the rodent, it has been found that hippocampal neurogenesis strongly recapitulates embryonic developmental trends (Esposito et al., 2005), and it seems likely that this would also be the case in primates. Because the length of embryonic neurogenesis is extended by a factor of about 6-10 in primates (6X in monkeys, 10X in humans), it makes sense that rates of neurogenesis and subsequent neural maturation are similarly extended (Kohler et al., 2011). Hence, and as emphasized by Kohler and colleagues, this longer period of neurogenic maturation is not completely unexpected and is likely appropriate for the preservation of neural plasticity over the longer life span of primates (Kohler et al., 2011).

Interestingly, and in contrast to that implied by the work of Kornak and Rakic (1999), some evidence also suggests that adult hippocampal neurogenesis in humans occurs at rates equal to or potentially greater than that which is seen in rodents. For example, Eriksson and colleagues injected human patients with a single dose of BrdU (a marker of neurogenesis) at rates equivalent to approximately 20mg/kg in rats. This is a very small dose that fails to detect upwards of 90% of mitotically dividing cells in rats (Snyder and Cameron, 2011). Furthermore, the patients in the Eriksson study were both chronically ill (and thus presumably experiencing a certain degree of stress) and advanced in age (mean age = 64 years), both of which serve to decrease rates of neural proliferation. Based on the results of Kornak and Rakic (1999), one might expect that the levels of neurogenesis in these human patients would be vanishingly low. However, when the brains of the patients were examined 6 months to 2 years after the initial BrdU injection, 25-325 BrdU+cells mm³ were found in the SGZ and GCL. Importantly, these levels fall within the range of healthy middle aged rats that have received multiple BrdU injections (Snyder and Cameron, 2011). This suggests that the rates of human neurogenesis might be at levels greater than that which is seen in rodents. Thus, it is clear that there is conflicting data regarding rates of hippocampal neurogenesis in humans. As such, it is advisable

to be wary of treating as equivalent neurogenesis-related findings in rodents with what is or might be the case in human populations.

1.4. Functional Consequence of Hippocampal Neurogenesis

The generation of new hippocampal neurons in adulthood is a feature that has been conserved throughout evolution, being found in every mammalian species examined to date including mice, rats, tree shrews, a variety of monkey species, and humans (Eriksson et al., 1998; Gould et al., 1997; Gould et al., 1999; Gould et al., 1999; Kornack and Rakic, 1999; Lerner et al., 2007). The evolutionary conservation of neurogenesis, in conjunction with the fact that neurogenic processes are actively regulated by psychological and environmental stimuli (see section 1.2) argues strongly against the possibility that neurogenesis is a mere evolutionary or developmental vestige. Instead, these results suggest that the generation of new dentate gyrus neurons in adulthood serves an important adaptive role in terms of hippocampal plasticity and function. Consistent with this, it has been established using a variety of different techniques that adult generated neurons functionally integrate into surrounding neural circuitry (Carlen et al., 2002; van Praag et al., 2002; Toni et al. 2008). Indeed, considerable support exists for a role of hippocampal neurogenesis in learning and memory processing. This is perhaps not surprising given the specific location of neurogenesis within the adult mammalian brain. Not only does neurogenesis occur in the hippocampus – a structure often conceptualized as the ‘gateway to memory’ – but also within the dentate gyrus, which is the narrowest spot in the tri-synaptic circuit of the hippocampus (i.e., entorhinal cortex to dentate gyrus, dentate gyrus to CA3, and CA3 to CA1; Kempermann, 2002). In this way, neurogenesis occurs in the region of the hippocampus through which all information must pass before it can be memorized. This bottlenecked location makes strategic sense from an evolutionary perspective as it readily allows for newly born neurons to contribute to the processing of environmental input and to hippocampal plasticity more generally. However, the precise functional consequence of hippocampal neurogenesis, even in terms of learning and memory, remains a subject of considerable research and debate.

In order to study neurogenesis, it is critical to have a method through which one is able to label neurons that have been generated in the adult brain. Perhaps because it readily crosses the blood-brain barrier and can thus be administered systemically (e.g., via intraperitoneal injection),

BrdU is the most commonly used marker of neurogenesis (Wojtowicz and Kee, 2006). BrdU is a thymidine analog, so it competes with endogenous thymidine for incorporation into mitotically dividing cells during DNA replication (i.e., the DNA synthesis phase, or S phase, of the cell cycle; Kee et al., 2007a). As such, BrdU essentially ‘tags’ those neurons that have recently undergone mitotic division; it marks the birth of the cell. This incorporation into the DNA of the cell is permanent and thus BrdU⁺ neurons can be detected for weeks, months, and even years after the initial injection using standard immunohistochemical procedures (Kee et al., 2007a). Importantly, by varying the delay between BrdU treatment and eventual sacrifice, it is possible to determine how adult generated neurons of different ages respond to environmental or psychological factors (e.g., a learning and memory scenario).

Another method used to investigate the role of adult neurogenesis in hippocampal function is to ablate newly generated neurons and observe the effect this has on subsequent hippocampal-dependent tasks. Methylazoxymethanol acetate (MAM) treatment prevents mitotically dividing cells from progressing through the cell cycle and is often implemented in these studies. For example, Shors and colleagues have shown that 14 days of MAM treatment in rats both downregulates adult neurogenesis and results in impaired learning of the hippocampal-dependent trace eye-blink conditioning task (Shors et al., 2001). Importantly, this MAM induced depletion in neurogenesis does not affect the ability of the rats to learn delay conditioning, a hippocampal-independent task. Furthermore, when rates of neurogenesis normalize 3 weeks post-MAM administration, so too does the ability of rats to learn the hippocampal-dependent trace conditioning. This result suggests that neurons aged 1-2 weeks play an important role in terms of mediating this hippocampal-dependent form of learning.¹ Importantly, a follow-up study by Shors and associates showed that the same MAM treatment failed to produce learning

¹ The work of Shors et al. (2001) clearly demonstrates that ablating 1-2 week old dentate granule neurons results in learning and memory deficits, indicating that even at this young age, the neurons are contributing to learning and memory. This result is particularly interesting in light of the work of Kee et al. (2007a) which has demonstrated that at 1-2 weeks of age, new neurons are not yet sufficiently mature to incorporate into a hippocampal-dependent memory trace. It is for future studies to determine how newly born cells improve learning without incorporating into the engram itself. One possibility is that at 1-2 weeks of age, newly generated neurons enhance the plasticity of neighboring neurons (e.g., by releasing some pro-plasticity molecule) thereby contributing to memory without incorporating into the memory trace itself. Consistent with this possibility, ablating neurogenesis decreases hippocampal plasticity more generally, as measured by long-term potentiation (Saxe et al., 2006).

deficits in contextual fear conditioning and spatial learning in the Morris Water Maze (MWM), two other hippocampal-dependent forms of learning and memory (Shors et al., 2002). However, others (e.g., Saxe et al., 2006; Winocur et al., 2006; Warner-Schmidt et al., 2008; Madsen et al., 2003) have shown that ablating neurogenesis through irradiation (at rates sufficient to diminish levels of neurogenesis but not other aspects of plasticity such as long-term potentiation; LTP) is sufficient to impair contextual fear conditioning in rodents. Furthermore, ablating neurogenesis negatively affects long-term memory retention in the MWM (Snyder et al., 2005; Deng et al., 2009) as well as performance on the Barnes maze (Raber et al., 2004), spatial working memory in the T-maze (Madsen et al., 2003), and hippocampal-dependent delayed non-matching-to-sample tasks (Winocur et al., 2006). Intriguingly, studies using irradiation (and also genetic ablation techniques) have also shown that adult hippocampal neurogenesis plays an important role in modulating the hippocampal-dependent period of associative fear memory (i.e., contextual fear conditioning; Kitamura et al., 2009). In particular, decreasing rates of neural proliferation results in a prolonged period throughout which the fear memory is dependent on the integrity of the hippocampus. In other words, adult neurogenesis seems to play a modulatory role in systems-level memory consolidation.

MAM and irradiation undoubtedly have effects on neural function that go beyond reductions in adult neurogenesis. With this, it is important to note that less-invasive genetic approaches that selectively ablate neural progenitors have also been developed and implemented. Results from these studies are consistent with an important role of neurogenesis in hippocampal-dependent learning and memory processing, although the details are (as above) still in need of clarification. For example, deficits in spatial memory in the MWM but intact contextual fear conditioning have been reported following genetic ablation of adult neurogenesis (Dupret et al., 2008; Zhang et al., 2008). Others, however, have reported the exact opposite pattern of results (i.e., intact spatial learning and memory with impairments in contextual fear conditioning; Saxe et al., 2006). The reasons for these discrepancies are uncertain, but likely involves differences in methodologies. For example, the time points at which rodents are tested following neurogenic depletion (Bruehl-Jungermann et al., 2007) and even slight variability in behavioural test apparatus settings (Aimone et al., 2011) can be critical when considering the functional role that adult generated neurons might play in cognitive processes.

That neurogenesis plays a functional role in hippocampal circuitry and subsequent learning and memory is further supported by the discovery that hippocampal-dependent learning tasks such as trace eye-blink conditioning and spatial learning in MWM enhance the survival of newly generated (1 week old) neurons, whereas hippocampal-independent tasks do not (Gould et al., 1999). This result demonstrates that hippocampus-dependent learning, not mere experience in the absence of learning or even learning that is independent of the hippocampus, enhances the survival of adult generated hippocampal neurons. Interestingly, the enhanced survival mediated by trace eye-blink conditioning correlates with improved ability to re-learn the paradigm up to 2 months after the initial training (i.e., a time at which the hippocampus is no longer required for the memory; Leuner et al., 2004). Furthermore, survival of newborn cells is greatest in rats that learn the MWM task well, compared to rats that learn poorly (Sisti et al., 2007). There is also a correlation between baseline rates of neurogenesis and the acquisition phase of the water maze task (Kempermann and Gage, 2002). In line with this, performance of aged rats in the MWM can be predicted based on the rats' baseline level of neurogenesis (Drapeau et al., 2003). As emphasized in an earlier section, environmental experiences that increase rates of adult neurogenesis (e.g., environmental enrichment or voluntary exercise) improve hippocampal-dependent learning and memory function (van Praag et al., 1999; Brown et al., 2003; Bruel-Jungerman et al., 2005), whereas experiences that decrease neurogenesis (e.g., chronic stress) result in deteriorated hippocampal-dependent learning and memory ability (Gould et al., 1992; 1997). In line with this, pharmacological compounds that specifically increase rates of neurogenesis also improve hippocampal-dependent learning and memory. For example, the compound P7C3 specifically enhances the survival of newly generated hippocampal neurons by inhibiting apoptotic cell death, and also enhances memory function in the MWM (Pieper et al., 2010). Interestingly, low levels of proliferation in hippocampus have been correlated with memory dysfunction in humans as well (Coras et al., 2010), thereby suggesting that the functional consequence of hippocampal neurogenesis has been conserved across rodent and primate species. Together, the results reviewed above strongly implicate adult hippocampal neurogenesis in specific forms of hippocampal-dependent learning and memory.

1.5. Unique Physiological Properties of Newly Generated Dentate Granule Neurons

The physiological mechanism through which newly generated hippocampal neurons endorse the formation of hippocampal-dependent learning and memory is currently unknown. There is, however, a plethora of evidence indicating that newly generated hippocampal neurons possess unique physiological properties, and it is possible that these unique properties might make them particularly well suited for involvement in hippocampal-dependent learning and memory (Bruehl-Jungman et al., 2007). For example, newly generated neurons display high levels of plasticity as indicated by their lower-thresholds for action potentials, LTP, and long-term depression (LTD; Massa et al., 2011; Mongiat et al., 2009; Schmidt-Hieber et al., 2004). In fact, induction of LTP in medial perforant path–granule cell synapses increases both proliferation and survival of newly generated (1-2 week old) granule neurons (Bruehl-Jungman et al., 2006; Chun et al., 2006) thereby indicating that LTP regulates neurogenic processes. Furthermore, relative to mature neurons, young dentate granule neurons are less sensitive to GABAergic inhibition (Ambrogini et al., 2004). Indeed, in very young granule neurons (i.e., < 3 week old), the inhibitory neurotransmitter GABA actually results in excitatory transmission (Ge et al., 2006, 2007, 2008). The initial over-expression of NKCC1 (a Cl^- importer, leading to high Cl^- content within the cell) on the dentate granule cell membrane is thought to underlie this initial depolarization (excitation) by GABA (Ge et al., 2006). At this early maturational time-point the neurons also possess higher resting membrane potentials, which likely accounts for their lower LTP thresholds (Esposito et al., 2005). These unique properties might allow the new neurons to differentially integrate into the surrounding neural circuit and make a disproportionately large contribution to learning and memory function (see section 1.8 for more information on memory allocation within neural circuits).

Adult-born hippocampal neurons begin integrating into the granule cell layer 4-10 days after generation and extend dendrites into the CA3 pyramidal cell layer another 4-10 days later. By approximately day 16, spines begin to appear on the dendrites, forming synapses with axon fibers (Deng et al., 2010; Toni et al., 2007; Zhao et al., 2006). This suggests that newly generated neurons form synapses long before they are fully mature (Ambrogini et al., 2004; Hastings and Gould, 1999; Toni et al., 2008). By 4-8 weeks post-generation, neurons have successfully integrated into surround circuitry (Carlen et al., 2002; van Praag et al., 2002), but they do not reach a fully mature morphology (soma size, total dendritic length, dendritic branching, and spine

density) until 4 months after their birth (Song et al., 2002). Indeed, during this 4-8 week period, the neurons display a competitive advantage and preferentially integrate into learning and memory circuitry (Kee et al., 2007a; refer to section 1.6 for more details). Around 4-6 weeks post-generation, the neurons exhibit robust synaptic plasticity, as indicated by their lower threshold for LTP and higher LTP amplitude (Ge et al., 2007). By having a subpopulation of hippocampal neurons that preferentially respond to inputs received while they are developing, the dentate gyrus will have a high probability of always possessing a subpopulation of neurons that will be capable of responding to any environment the animal experiences (Aimone et al., 2009, 2011). Computational models suggest that having a subpopulation of hyper-excitable immature neurons that readily respond to environmental input helps the system avoid ‘catastrophic interference’ (Appleby and Wiskott, 2009; Wiskott et al., 2006). That is, neurogenesis might allow the hippocampus to minimize the interference (and hence forgetting) that occurs with new learning by allowing mature neurons to stay adapted to previously learned information, while new neurons encode the more novel information found in subsequent learning scenarios. Indeed, it is this rationale that underlies the role neurogenesis plays in pattern separation (Aimone et al., 2011). In summary, the unique properties of adult generated hippocampal neurons might form the basis upon which neurogenesis preferentially contributes to hippocampal functionality.

1.6. Accelerated Functional Integration of 4-8 Week Old Dentate Granule Neurons

In line with the unique physiological characteristics possessed by immature neurons, evidence indicates that newly generated hippocampal neurons encode significant features of their environments (Kee et al., 2007a; Tashiro et al., 2007). For example, work from the Frankland lab has demonstrated that by the time adult-generated neurons are 4-8 weeks of age, they are 2-3x more likely to be recruited into spatial memory circuitry, relative to mature dentate granule neurons (Kee et al., 2007a). Conversely, 1-week old neurons do not integrate at all, and 2 week old neurons integrate at or below baseline levels. The molecular mechanisms underlying this accelerated functional integration into memory circuitry is currently unknown. What is ‘special’ about immature dentate granule neurons that underlies their preferential recruitment into memory circuitry? One method of shedding light on the mechanism underlying this competitive advantage would be to compare the intracellular properties of dentate granule neurons (of

different levels of maturation) in response to memory retrieval. In particular, there is enticing evidence suggesting that the intracellular protein cyclic adenosine monophosphate (cAMP) responsive element-binding protein (CREB) might play a critical role in mediating this ‘preferential recruitment’ effect. For example, CREB has been implicated in mediating many stages of neurogenesis (e.g., proliferation, maturation, survival; Jagasia et al., 2009; Nakagawa et al., 2002ab). Furthermore, CREB activation has recently been shown to regulate the intrinsic excitability of neurons in many neural regions (for an insightful review, see Benito and Barco, 2010). As such, that CREB might modulate the competitive advantage and preferential incorporation into spatial memory circuitry experienced by immature neurons (4-8 weeks old) is a compelling possibility. The evidence in favor of this possibility is discussed in greater detail below.

1.7. Role of CREB in Intrinsic Neuronal Excitability

CREB is a transcription factor that has long been known to be involved with long-term memory consolidation. Specifically, the activation of CREB in response to synaptic activity elicited by a learning experience is required to induce the molecules necessary for the structural stabilization (i.e., strengthening) of the memory trace that leads to long-term retention (Bartsch et al., 1995; Bourtchuladze et al., 1994; Brunelli et al., 1976; Byers et al., 1981; Dudai et al., 1983; Kandel et al., 2001; Silva et al., 1998; Yin et al., 1994; Yin et al., 1995). In short, CREB has been established as a key molecular switch that converts short-term into long-term memory. However, recent studies suggest a novel role for CREB in neural plasticity that broadens its physiological function beyond long-term memory consolidation. In particular, recent evidence suggests that CREB plays a role in modulating the intrinsic excitability of neurons, that is, the propensity of a neuron to fire an action potential (i.e., become active) in response to input (Benito and Barco, 2010). Here, it is important to note that intrinsic excitability is distinct from synaptic plasticity, with the former involving changes in the electrical properties of an individual neuron and the latter involving changes at the synapse between neurons (Benito and Barco, 2010; Mozzachiodi and Byrne, 2010).

Dong and colleagues were the first to directly examine the potential role of CREB in modulating intrinsic neural excitability. By genetically manipulating the activity of CREB in neurons of the nucleus accumbens, these researchers discovered that overactivating CREB

enhanced the intrinsic excitability of neurons, while the expression of a dominant negative CREB reduced it (Dong et al., 2006). Neuronal excitability is ultimately determined by the properties and distribution of ion channels on the cell membrane (Daoudal and Debanne, 2003). Consistent with this, Dong and colleagues found that CREB influences intrinsic neuronal excitability by influencing voltage-gated Na^+ and K^+ channels. Specifically, CREB overactivation leads to an increase in the influx of Na^+ currents and a decrease in the efflux K^+ currents (thereby depolarizing the cell), whereas CREB inhibition has the opposite effect. Manipulating CREB activity also lowered the threshold for an action potential (i.e., the minimum current required to fire a spike; Dong et al., 2006). Further, CREB function has been manipulated in neurons in the locus coeruleus, whereby genetically overactivating CREB results in increased intrinsic excitability and a more depolarized resting membrane potential, whereas inhibiting CREB activity has the opposite effect (Han et al., 2006). The intrinsic excitability of CA1 pyramidal neurons are also modulated by genetic manipulations of CREB activity (dentate granule neurons have not yet been examined). Enhancing CREB activity in these neurons both increases neuronal excitability and inhibits afterhyperpolarization (i.e., a current that prevents the same neuron from firing again, thereby reducing neuronal excitability; Lopez de Armentia et al., 2007). CREB inhibition has the opposite effect, reducing the number of action potentials elicited by a depolarizing current. Enhancing CREB activity also produces an increase in spike frequency and a reduction in afterhyperpolarization in neurons in the basal nucleus of the amygdala (Viosca et al., 2009), as well as a reduction of spike threshold and increased probability of action potentials in lateral amygdala neurons (Zhou et al., 2009). Finally, and as reviewed elsewhere (Benito and Barco, 2010), results obtained in *Drosophila melanogaster* also suggest a role of the CREB signalling pathway in regulating ion currents and ultimately neuronal excitability. Together, these results strongly implicate CREB activity as a critical and evolutionarily conserved mechanism through which intrinsic neuronal excitability is regulated across a variety of animal species and neuronal types.

1.8. Memory Allocation as a Competitive Process: Role of CREB and Intrinsic Neuronal Excitability

Little is known about how the brain allocates information within neural circuitry or the mechanisms that determine which neurons are recruited in response to a learning or memory

episode. Memory allocation might involve some sort of competition between individual neurons and synapses. Conversely, there might not be a strict mechanism underlying the process by which neurons participate in a memory trace – it might largely be random. Interestingly, evidence suggests that the former possibility is more likely. As discussed above, recent work suggests that CREB plays an important role in regulating intrinsic neuronal excitability. Importantly, intrinsic excitability likely serves as a molecular mechanism through which a neuron can be set into a permissive state, ready to participate in a given memory trace (Mozzachiodi and Byrne, 2010). Thus, the role of CREB in regulating intrinsic neuronal excitability represents a significant breakthrough and has important implications in terms of the allocation of information within learning and memory circuitry. For instance, if a neuron is more likely (relative to surrounding neurons) to fire an action potential in response to environmental stimuli, it will be at the same time more likely to incorporate into the memory circuit responsible for representing said environmental stimuli. Experimental evidence is consistent with a role for CREB in memory allocation. For example, artificially increasing CREB in a subset of lateral amygdala neurons results in an approximately 3-fold increase in probability that these neurons participate in a fear memory for a tone (Han et al., 2007). This result is particularly interesting and suggests that enhanced CREB activity confers a competitive advantage to a neuron, rendering it more likely to participate in a fear memory trace. Thus, by regulating intrinsic neuronal excitability, CREB also seems to be involved with memory allocation – the set of processes that determine where information is stored within a neural circuit (Silva et al., 2009).

A targeting lesion strategy has further confirmed the role of CREB in memory allocation. In this study, the researchers artificially increased levels of CREB in a subset of lateral amygdala neurons, resulting in an approximately 3-fold increase in probability that these neurons participated in a fear memory for a tone (i.e., the same result found by Han et al., 2007). The critical manipulation in this study is that following the learning episode, the CREB-enhanced neurons were selectively deleted via an inducible diphtheria-toxin strategy. The researchers reasoned that if the CREB-enhanced neurons truly play a preferential role in the allocation of the memory, then deleting these neurons following the initial learning period should have a disproportionately negative impact on the integrity of the memory. Indeed, as predicted, deleting the amygdala neurons that overexpressed CREB after the learning episode selectively deleted the fear memory for the tone. Conversely, deleting a similar proportion of random amygdala neurons

not overexpressing CREB did not affect the expression of fear memory. This deletion was remarkably specific, as ablating these CREB-enhanced neurons did not affect previously acquired memories, nor did it disrupt the ability of the animals to form new amygdala-dependent memories (Han et al., 2009). This result convincingly demonstrates that CREB modulates memory allocation. Neurons with greater CREB activation out-compete and are preferentially recruited into the memory trace (Josselyn, 2010). Importantly, Zhou and associates have demonstrated that following tone conditioning, the potentiation of lateral amygdala inputs is larger in the transfected CREB-enhanced neurons relative to control neurons (Zhou et al., 2009). This result is consistent with CREB playing a modulatory role in intrinsic neuronal excitability and suggests that such modulation serves as the substrate for the competitive advantage and preferential recruitment seen in CREB-enhanced neurons. To date, the extent to which CREB regulates memory allocation outside of the lateral amygdala is unknown.

1.9. CREB and the Preferential Recruitment of 4-8 week Old Dentate Granule Neurons into Memory Circuitry

The results summarized above help answer the question of why certain neurons, rather than their neighbors, are recruited into a given memory trace. As discussed above (sections 1.5 and 1.6), immature granule neurons possess a high level of intrinsic excitability allowing them to make a unique contribution to learning and memory circuitry (section 1.4). Neurobehavioural models (section 1.4 – 1.6) have shown that immature dentate granule neurons are more likely to be recruited into a spatial memory trace, which is to say that adult generated neurons play a special role in memory allocation (section 1.8). The molecular mechanisms underlying the preferential recruitment of immature hippocampal neurons into memory circuitry is unknown. CREB regulates intrinsic excitability in a variety of neural regions and plays a role in memory allocation in the lateral amygdala. Interestingly, immature hippocampal neurons express pCREB (the phosphorylated and ‘active’ version of CREB), with increased levels of this protein correlating with both proliferation, maturation, and survival of adult generated neurons (Fujioka et al., 2003; Jagasia et al., 2009; Nakagawa et al., 2002ab). This suggests that, in addition to its role in the amygdala, CREB may be an important factor in regulating neurogenic dependent memory processes in the hippocampus. However, the potential role of CREB in regulating the

accelerated functional integration and preferential recruitment of immature hippocampal neurons into memory circuitry (i.e., the results of Kee et al., 2007a) has not yet been investigated.

1.10. Current Experiment

In the current study, a key question is being addressed: what accounts for the competitive advantage and preferential recruitment of immature dentate granule neurons into spatial memory circuitry? What is special about these newly generated neurons that accounts for their unique contribution to memory allocation? Given the role of CREB in regulating neurogenic processes (e.g., proliferation, maturation, and survival) in conjunction with its role in mediating intrinsic neural excitability (and fear memory allocation in the lateral amygdala), one compelling possibility is that activated CREB also plays an important role in regulating the functional integration of newly generated adult neurons. To address this possibility, replicating granule cells were labeled with BrdU at a variety of time-points before training rats in a MMW task. This allowed the determination of how granule cells of different ages and levels of maturation respond to a spatial learning and memory scenario. To investigate whether CREB plays a role in mediating the competitive advantage and preferential recruitment effect, we examined whether 6 week old dentate granule neurons preferentially expressed the phosphorylated (and active) version of CREB, relative to both neighboring neurons and younger (1-3 week old) BrdU⁺ cells. Double-label immunofluorescence for BrdU and pCREB in conjunction with high-resolution confocal microscopy was used to determine whether 6 week old neurons preferentially express pCREB relative to average mature neurons in response a spatial learning and memory experience.

CHAPTER 2

METHODS

2.1. Subjects

This experiment was performed on male Long Evans rats (Charles River Laboratories, Quebec, Canada; n=48; weight=200g upon arrival). The rats were pair housed with free access to both food and water. The animal colony room was maintained at 21±2°C with a 12h light-dark cycle with lights on at 08:00h. All experimental manipulations occurred during the light cycle. Upon arriving in the colony, rats were given 3 days to acclimate. Each rat was then handled for

approximately 2 minutes for 5 consecutive days prior to any experimental manipulations (i.e., BrdU injections). This work was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

2.2. BrdU Administration

BrdU was dissolved in saline heated to 50-60°C and then filtered. The BrdU (100mg/kg) was injected intraperitoneally twice a day for 5 consecutive days. Because the S-phase of the cell cycle lasts approximately 8 hours (Kee et al., 2007b), it was ensured that at least 8 hours passed in between each injection such that distinct populations of dividing cells were labeled with each injection. Following the general protocol laid out by Frankland and colleagues (Kee et al., 2007ab), BrdU was injected into rats either 1, 3, 6, or 10 weeks prior to training on the MWM (n=8 per group) (see Figure 2.1).

2.3. Water Maze Procedure

Behavioural testing was conducted in a circular water maze tank (2 meters in diameter) located in a room lit dimly by four lamps. The pool was filled to a depth of 29.5cm and made opaque with white, non-toxic powdered paint. The water was kept at a temperature of 27-29°C. A square escape platform (15 x 15cm) was submerged 4cm below the surface of the water and maintained in a fixed location (30cm from the wall of the pool) in one of the quadrants. The water maze was surrounded by white curtains that had distinct black cues attached in 3 of the 4 quadrants. The curtains were positioned approximately 1 meter away from the perimeter of the water maze.

Rats (n=32) received 4 training trials a day for 5 consecutive days. On each trial, the rat was placed into the pool, facing the wall, in one of four start locations (N, S, E, or W). The start location was varied throughout the training days. A single training trial consisted of the rat swimming freely in the water maze. If the rat failed to locate the hidden platform after 60s had elapsed, it was gently guided to the platform by the experimenter. The rat remained on the platform for 15s before being removed by the experimenter. The intertrial interval between the four training trials was approximately 4min. The probe test was performed on the day following

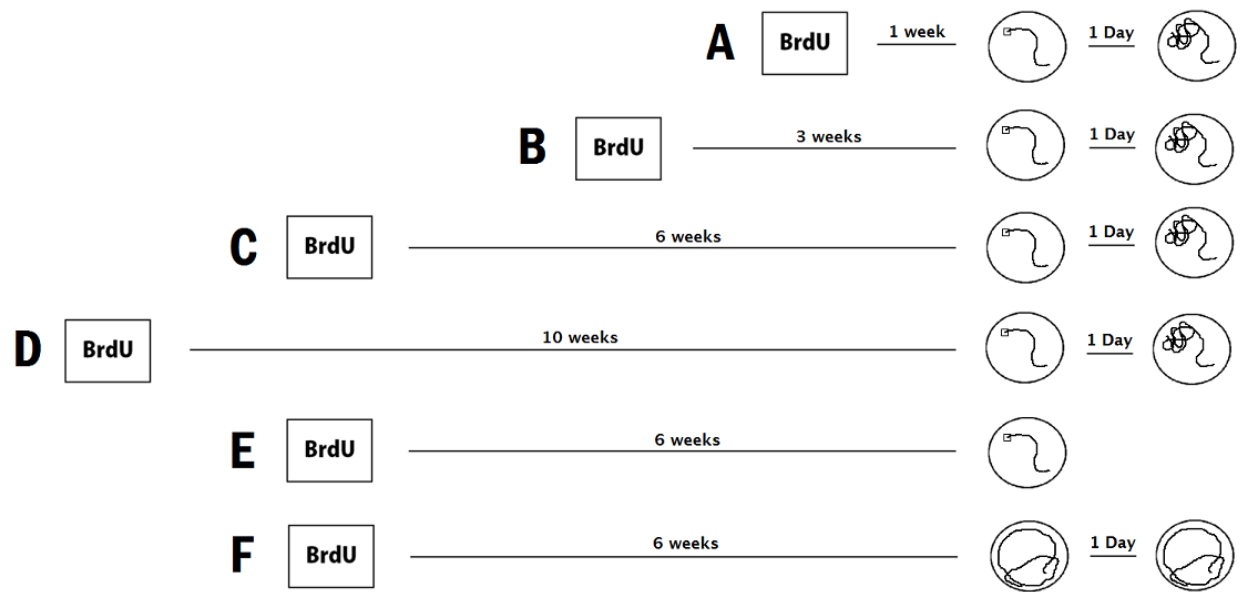


Figure 2.1. Experimental design of the current study. Full five-day training on the water maze began (A) 1 week, (B) 3 weeks, (C) 6 weeks, or (D) 10 weeks following BrdU administration. The probe test occurred one day following completion of training. (E) A cohort of rats injected with BrdU 6 weeks earlier engaged in a single-session rapid learning protocol on the water maze. (F) A cohort of rats injected with BrdU 6 weeks earlier engaged in swimming (i.e., no escape platform) on both the five training days and the probe test day. Note that a cohort of the 6 week rats were never exposed to the water maze and instead served as cage-controls.

the last day of training. Here, the platform was removed from the water maze and the rat was allowed to swim freely for 60s before being removed from the water. The time spent in the quadrant previously occupied by the escape platform was used as an index of memory for where the platform was previously located.

A cohort of rats (n=8) injected with BrdU 6 weeks before training were subjected to single-session learning protocol that has been shown previously to form robust memory for the location of the platform (Wong et al., 2007; Ge et al., 2010). This protocol readily captures the initial learning or acquisition phase of spatial memory formation (i.e., relative to a 5 day learning protocol followed by a probe test on the 6th day). The rats being trained with this protocol underwent 8 training trials in rapid succession (intertrial interval=30s), using the following start points: [N, S, E, W, S, N, W, E]. As before, rats were allowed to swim freely for 60s or until they found the hidden escape platform. If the rat failed to locate the hidden platform after 60s had elapsed, it was gently guided to the platform by the experimenter. The rat remained on the platform for 15 s before being removed from the pool.

There was also a group of rats injected 6 weeks prior to experimental manipulation. These animals served as either swimming controls (n=4) or cage controls (n=4). The ‘swim only’ animals swam in the water maze without the platform and without the distinct black cues on the curtain surrounding the water maze. These rats swam in the water maze for 60s, 45s, 30s, 15s, and 15s per trial on training day 1, 2, 3, 4, and 5, respectively. There were 4 training trials each day, with the inter-trial interval set at approximately 4min. These rats were also tested in the 60s probe test (memory not expected) such that their experience would be as similar as possible to the animals that were actually trained on the task. The data of these ‘swim only’ animals is not reported here as animals, in the absence of training, do not show memory for the platform.

For all probe trials, the water maze was conceptually divided into four equal quadrants (NW, NE, SW, SE). As has been reported elsewhere (e.g., Kee et al., 2007a; Wright and Conrad, 2008), time spent in the target quadrant (i.e., the quadrant that previously housed the escape platform) was compared to time spent in the opposite quadrant. Time spent swimming in the target quadrant is used as an index of memory for where the escape platform was previously located. All training and probe trials were recorded with a videocamera and analyzed using an EthoVision tracking system (Noldus).2.4. Tissue Preparation

Rats were sacrificed and transcardially perfused 3 hours following completion of the MWM testing, as this time point coincides with pCREB protein expression in response to the behavioural task (Bilang-Bleuel et al., 2002; Cammarota et al., 2000; Countryman et al., 2005; Kuipers et al., 2006; Mamiya et al., 2009; Roozendaal et al., 2006). The rats were deeply anesthetized using Euthanyl Forte and perfused with saline followed by chilled 4% paraformaldehyde. Brains were removed and post-fixed in paraformaldehyde for three days. At this time, the brains were transferred into a PBS solution containing 0.02% sodium azide and stored at 4°C until later processing. Brains were sectioned in twelve sets of 50µm sections and covered the entire hippocampus.

2.5. BrdU+pCREB Double-Label Immunofluorescence

Immunohistochemical analysis was performed on randomly chosen animals from the 1 week (n=4), 3 week (n=4), 6 week (n=4), 6 week 'learn-only' (n=4), 6 week swim-only (n=3), and 6 week cage control animals (n=4). Analysis was not performed on the 10 week group due to limited primary immunohistochemical reagents.

Immunohistochemistry was performed on free-floating sections with all rinses and incubations carried out under gentle agitation. The sections were first rinsed in TBS and then antigen retrieval (sodium citrate) at 80°C for 30min. After cooling to room temperature, the sections were rinsed with TBS and then incubated in 1N HCl at 45°C for 40min. The sections were then exposed to 0.1M borate buffer for 12min. Following TBS rinses, the sections were placed in a blocking solution containing 5% (v/v) normal donkey serum, 1% (w/v) bovine serum albumin, and 0.3% (v/v) Triton-X 100 in TBS for 60min at room temperature. After blocking, the sections were incubated for 24 hours at room temperature in BrdU (mouse anti-BrdU monoclonal antibody, 1:100, Roche Applied Sciences) and pCREB [ser133] (rabbit anti-pCREB polyclonal antibody, 1:2000, Millipore) or NeuN (mouse anti-BrdU monoclonal antibody, 1:1000) and pCREB [ser133] (rabbit anti-pCREB polyclonal antibody, 1:2000, Millipore). Following TBS rinses, the sections were incubated in 1:500 of Alexa-488 [green fluorescence] donkey anti-rabbit and 1:500 of Alexa-568 [red fluorescence] donkey anti-mouse (diluted in 0.3% Triton-X 100 in TBS) for three hours at room temperature. Sections were mounted on glass slides and coverslipped with the mounting medium citifluor.

2.6. Quantification

All fluorescence images were captured using a Zeiss LSM 700 confocal imaging microscope equipped with a 405 nm, 488 nm, 555 nm and 639 nm solid state laser lines. A Zeiss Plan-Apochromat 63x/1.40 oil DIC objective lens was used to obtain images for quantitative analysis. A 10x and 20x dry objective confocal lens (W Plan-Apochromat 10x and 20x/1.0 DIC lens) were also used to obtain overview images of the GCL. Laser intensities, detector sensitivity, and digital offset were held constant throughout the images. All images were taken using the multi-track channel to prevent bleed through. The pixel size for all images was set to 0.06 μ m by 0.06 μ m.

The purpose of the current study is to determine whether immature dentate granule neurons preferentially express pCREB relative to average mature dentate granule neurons in response to a learning and memory situation. To accomplish this goal, the difference between pCREB expression in BrdU⁺ vs BrdU⁻ cells was calculated, with positive scores indicating greater pCREB expression in the BrdU⁺ cells (Table 3.1). All images were obtained by an experimenter blind to the conditions from which the tissue came. The entire anterior-posterior extent of the dentate gyrus was examined. Only cells within the GCL were investigated in the current study. pCREB immunoreactivity was quantified using optical densitometry (ImageJ software) from approximately 4 BrdU⁺ and 4 BrdU⁻ cells from each hippocampal section across all groups examined. This resulted in approximately 20 BrdU⁺ and 20 BrdU⁻ neurons (min=15, max=30) examined per animal. This allowed us to determine the average pCREB expression in adult generated dentate granule neurons (of various ages) in response to training on the MWM.

As emphasized above (section 1.3), almost all adult-generated cells found in the GCL are neuronal in lineage (see Figure 3.7 and 3.8; Cameron et al., 1993; Gage et al., 1995). As such, only cells in the GCL were included in the current analysis. In conjunction with this, emphasis was placed on the morphological characteristics of the cells, such that only neurons would be included in the final analysis. Glia were excluded based on size (generally smaller than granule neurons) and shape (neurons are circular, whereas glial cells are often possess a triangular or irregular shape; Cameron et al., 1993; Parent et al., 1997; Rapp and Gallagher, 1996). Thus, although neuron-specific markers were not used in the current study, we are confident that all cells included in the final analysis were neuronal in lineage based on their location in the hippocampus and morphological characteristics (see Figure 3.9 and 3.10).

CHAPTER 3

RESULTS

3.1. Water Maze Results

A 4x5(BrdU date x training day) mixed factor ANOVA was used to assess performance on the MWM across the different training days and to examine whether the timing of BrdU injection influenced this performance. The time required to locate the platform was used as an index of learning and memory, with reduced latencies being indicative of superior performance. As expected, there was a significant effect of training day [$F(5,119) = 271.308$, $p < 0.001$], indicating that the performance of the rats improved with increased training (i.e., the rats learned the task; Figure 3.1.). Post-hoc Tukey HSD tests revealed that MWM performance on training day 1 was significantly worse than performance on all subsequent training days [all p 's < 0.001]. Likewise, performance on training day 2 was significantly worse than performance on training days 3, 4, and 5 [p 's < 0.005]. And finally, performance on training day 3 was worse than performance on training day 5 [$p = 0.001$]. In sum, MWM performance improved across training days, with the rats requiring progressively less time to locate the escape platform with training (Figure 3.1).

Importantly, the time at which rats received BrdU injections prior to training did not influence overall performance on the MWM [$F(15,363) = 1.426$, $p = 0.132$]. There was, however, a significant interaction between time of BrdU injection and performance across training days on the MWM, specifically on the first training day [$F(3,123) = 3.468$, $p = 0.018$]. Tukey's HSD post-hoc tests revealed that the 6 week group [$M=53.75s$] performed significantly worse than the 1 week group [$M=39.37s$] on the first training day [$p = 0.011$]. This difference disappeared by the second training day. There were no other significant differences in MWM performance across days (Figure 3.2).

To examine probe trial performance, we first examined whether there were differences in performance based on time of BrdU injection. As expected, a one-way ANOVA failed to find a significant difference in time spent in the target quadrant based on time of BrdU injection [$F(3,44) = 0.552$, $p = 0.651$]. Consistent with this, paired-samples t-tests revealed that the one week rats [$t(7) = 4.885$, $p = 0.002$], three week rats [$t(7) = 3.423$, $p = 0.011$], six week rats

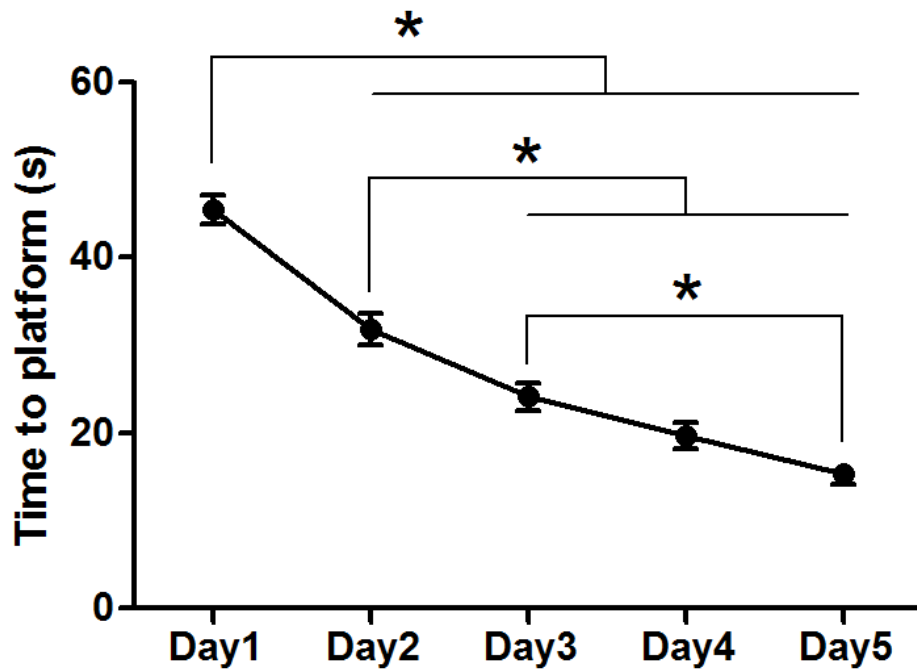


Figure 3.1. The effect of training on MWM performance. As expected, performance improved as a function of days spent training [$p < 0.001$]. Post-hoc tests revealed that performance on training day 1 was significantly worse than performance on all subsequent training days [all p 's < 0.001]. Likewise, performance on training day 2 was significantly worse than performance on training days 3, 4, and 5 [all p 's < 0.005]. And finally, performance on training day 3 was worse than performance on training day 5 [$p = 0.001$]. Error bars denote S.E.M.

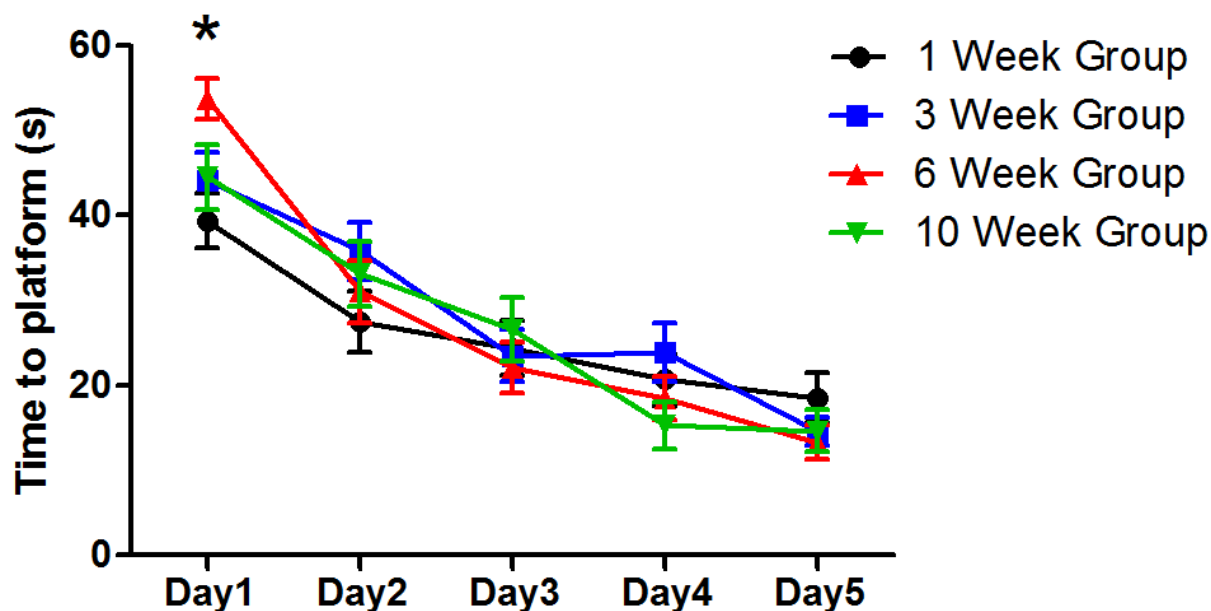


Figure 3.2. Performance on the MWM across days as a function of time of BrdU administration. The time of BrdU injection did not affect overall performance in the water maze [$p = 0.132$]. However, there was a statistically significant interaction between time of BrdU administration and performance across days on the MWM [$p = 0.018$], an effect driven by the difference in performance of the 1 week and 6 week groups on the first day of training [$p = 0.011$]. This effect disappeared by the second training. There were no other significant differences in MWM performance across days, as a function of time of BrdU injection. Error bars denote S.E.M.

[$t(7) = 5.090$, $p = 0.001$], and 10 week rats [$t(7) = 5.182$, $p = 0.001$] all spent more time searching for the escape platform in the target quadrant versus the opposite quadrant (see Figure 3.3). Because the probe trial performance of all groups was equivalent, the probe data were collapsed and analyzed together. This collapsed probe trial data showed that the rats spent significantly more time in the target quadrant [Mean=25.72s] than in the opposite quadrant [Mean=11.37s], [$t(31) = 8.933$, $p < 0.001$], thereby suggesting that the animals successfully learned and remembered the location of the escape platform (Figure 3.4).

A repeated measures ANOVA was used to assess MWM performance in the rapid learning group. As anticipated, the rats successfully learned the location of the platform across trials [$F(7,56) = 3.502$, $p = 0.003$]. Post-hoc Tukey tests found statistically significant differences between training trials 2 and 5, 6, and 8 [p 's < 0.031] and a near-significant difference between trials 2 and 7 [$p = 0.064$]. A clear trend towards improved performance across training trials was present (Figure 3.5). Consistent with this, a paired samples t-test comparison between the first 4 training trials [M=42.57s] and the last 4 training trials [M=22.51s] was significant, [$t(31) = 3.78$, $p = 0.001$], indicating that the performance of the rats improved across the training session (Figure 3.6). This result suggests that the rats successfully learned the location of the escape platform over the 8-trial rapid learning paradigm.

3.2. BrdU+pCREB Immunofluorescence

Photomicrographs of pCREB and NeuN immunoreactive cells in the GCL at 10x and 20x are found in Figure 3.7 and 3.8, respectively. Photomicrographs of BrdU⁺ cells in the GCL at 20x and 63x magnification are found in Figure 3.9 and 3.10, respectively. The mean pCREB expression data obtained from both BrdU⁺ and BrdU⁻ cells along with the differential score calculated from these two cells types (i.e., the values used in statistical analysis) is reported in Table 3.1.

Using Figure 3.11 as a reference, qualitative analysis indicates that 1 week old BrdU⁺ neurons display the lowest level of pCREB expression relative to average GCL neurons (i.e., their differential score is negative). In dramatic contrast, 3 week old neurons display the highest level of pCREB expression in response to learning and memory on the MWM task. The expression of pCREB in 6 week old dentate granule neurons in response to both full training on

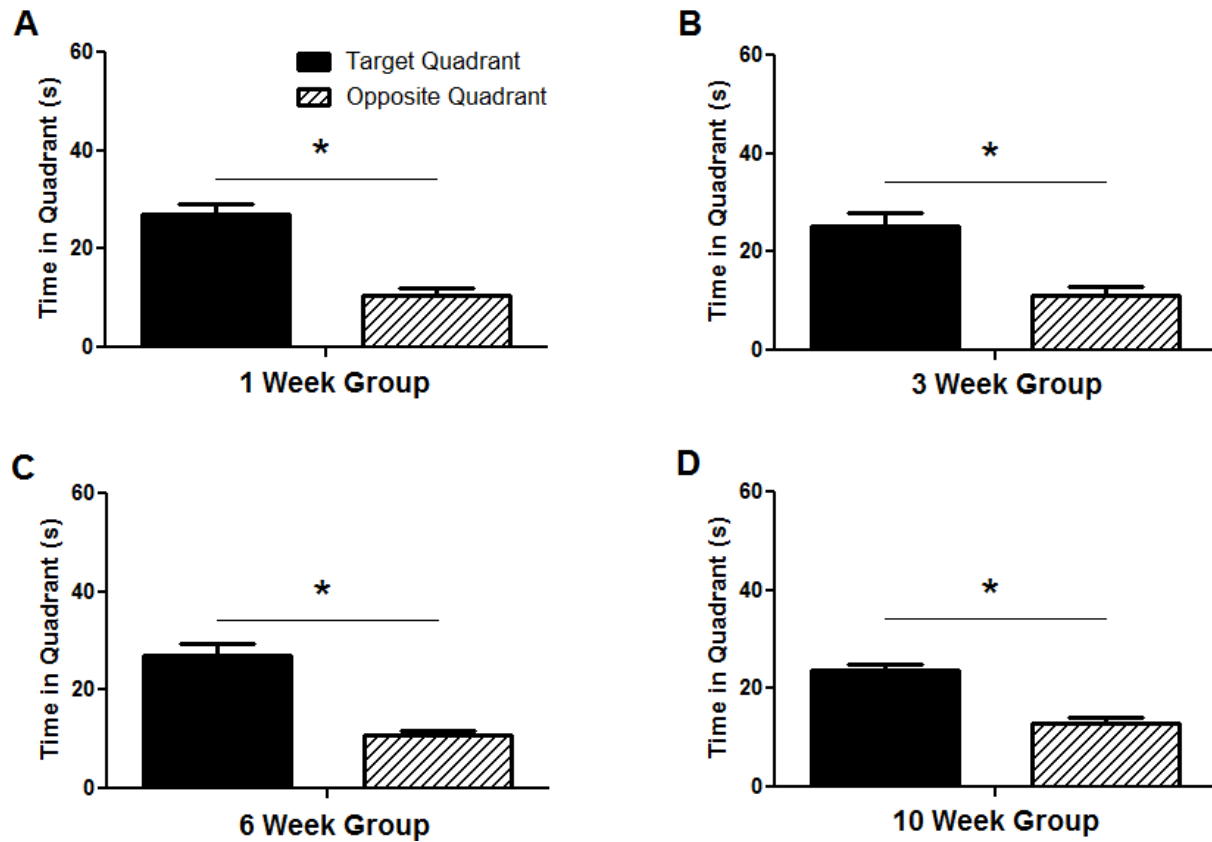


Figure 3.3. Effect of BrdU administration of probe trial performance. (A) Rats that received BrdU injections 1 week prior to MWM training spent more time searching the target quadrant [$p = 0.002$]. (B) Rats that received BrdU injections 3 weeks prior to MWM training spent more time searching the target quadrant [$p = 0.011$]. (C) Rats that received BrdU injections 6 weeks prior to MWM training spent more time searching the target quadrant [$p = 0.001$]. (D) Rats that received BrdU injections 10 weeks prior to MWM training spent more time searching the target quadrant [$p = 0.001$]. Thus, regardless of time of BrdU injection (i.e., 1, 3, 6, or 10 weeks prior to MWM training), the rats spent more time searching the target quadrant compared to the opposite quadrant. Error bars denote S.E.M.

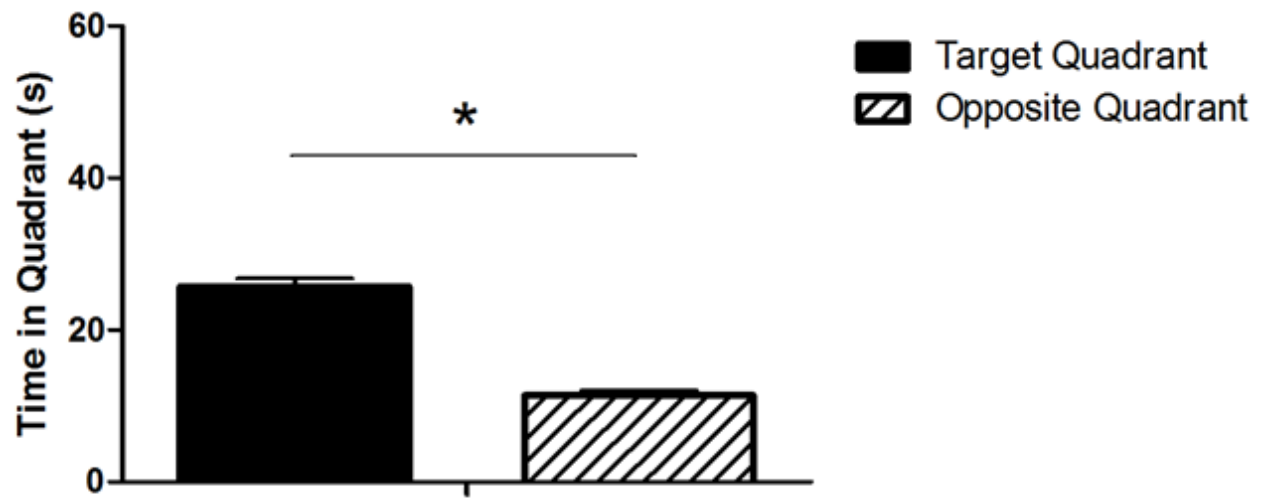


Figure 3.4. Overall probe trial performance. As expected, the rats spent more time in the target quadrant [Mean=25.72s] than in the opposite quadrant [Mean=11.37s] [$p < 0.001$]. This result indicates that the animals successfully learned and remembered the location of the escape platform. Error bars denote S.E.M.

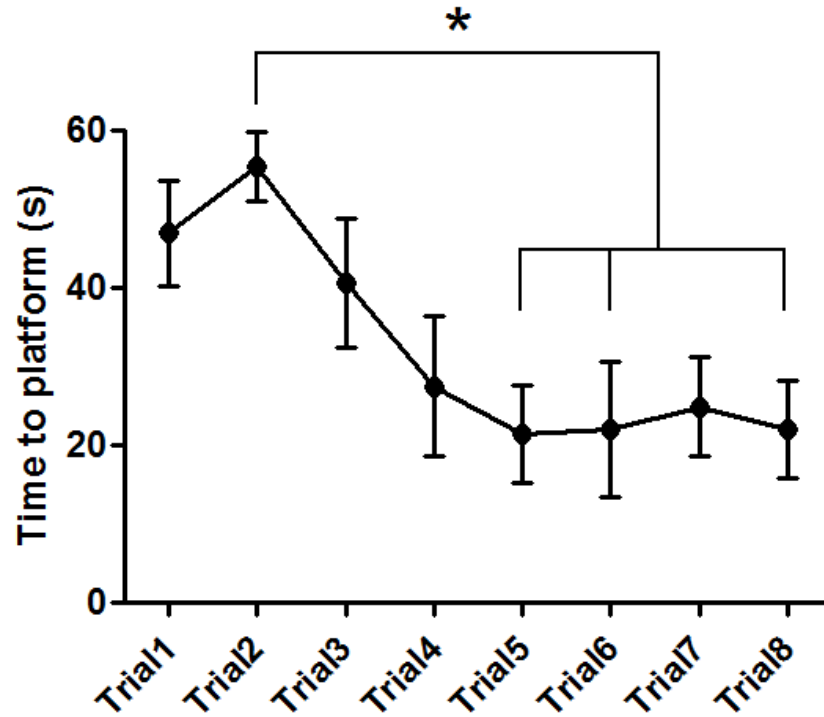


Figure 3.5. MWM performance in the 8-trial single-session rapid learning condition. There was a significant effect of training trial [$p = 0.003$], suggesting that the animals successfully learned the location of the escape platform. Post-hoc analysis indicated that the rats performed better on trials 5, 6, and 8 than on trial 2 [p 's < 0.031]. The difference in performance between trial 2 and 7 was nearing statistical significance [$p = 0.064$]. There is a clear trend towards improved performance across the 8 training trials. Error bars denote S.E.M.

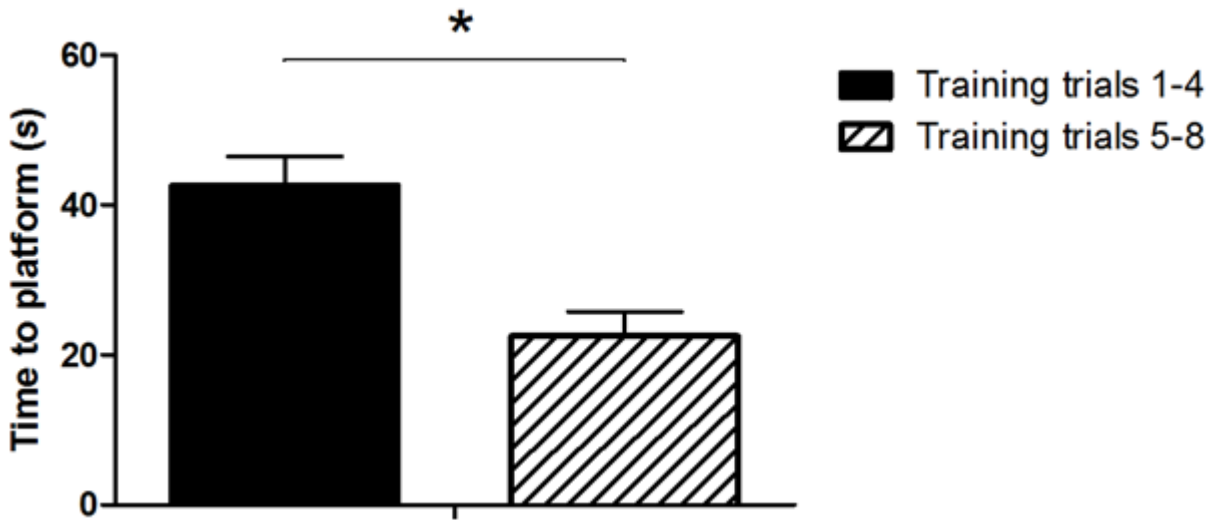


Figure 3.6. Comparison of MWM performance during first 4 trials and last 4 trials in the rapid learning condition. The performance of the rats was better in the last 4 trials [$p = 0.001$], suggesting that the rats successfully learned the location of the escape platform over the 8 training trials. Error bars denote S.E.M.

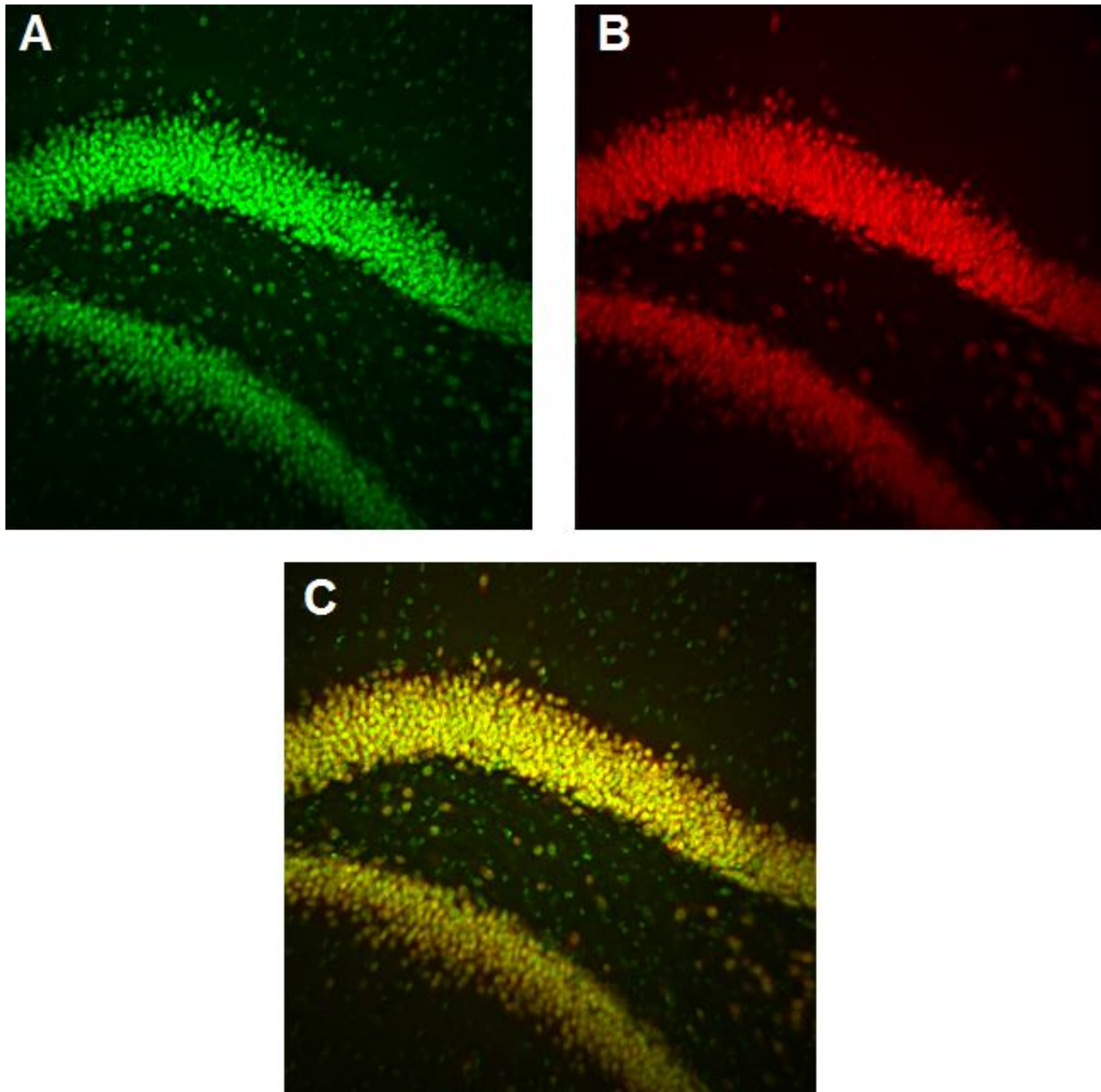


Figure 3.7. Photomicrographs of pCREB and NeuN immunoreactive cells in the GCL at 10x magnification. (A) pCREB immunoreactivity [green] in the GCL. (B) The corresponding NeuN⁺ cells [red] in the GCL. (C) Merged image of pCREB and NeuN⁺ cells [yellow] in the GCL. Note that almost all GCL cells are neuronal in lineage (i.e., NeuN⁺).

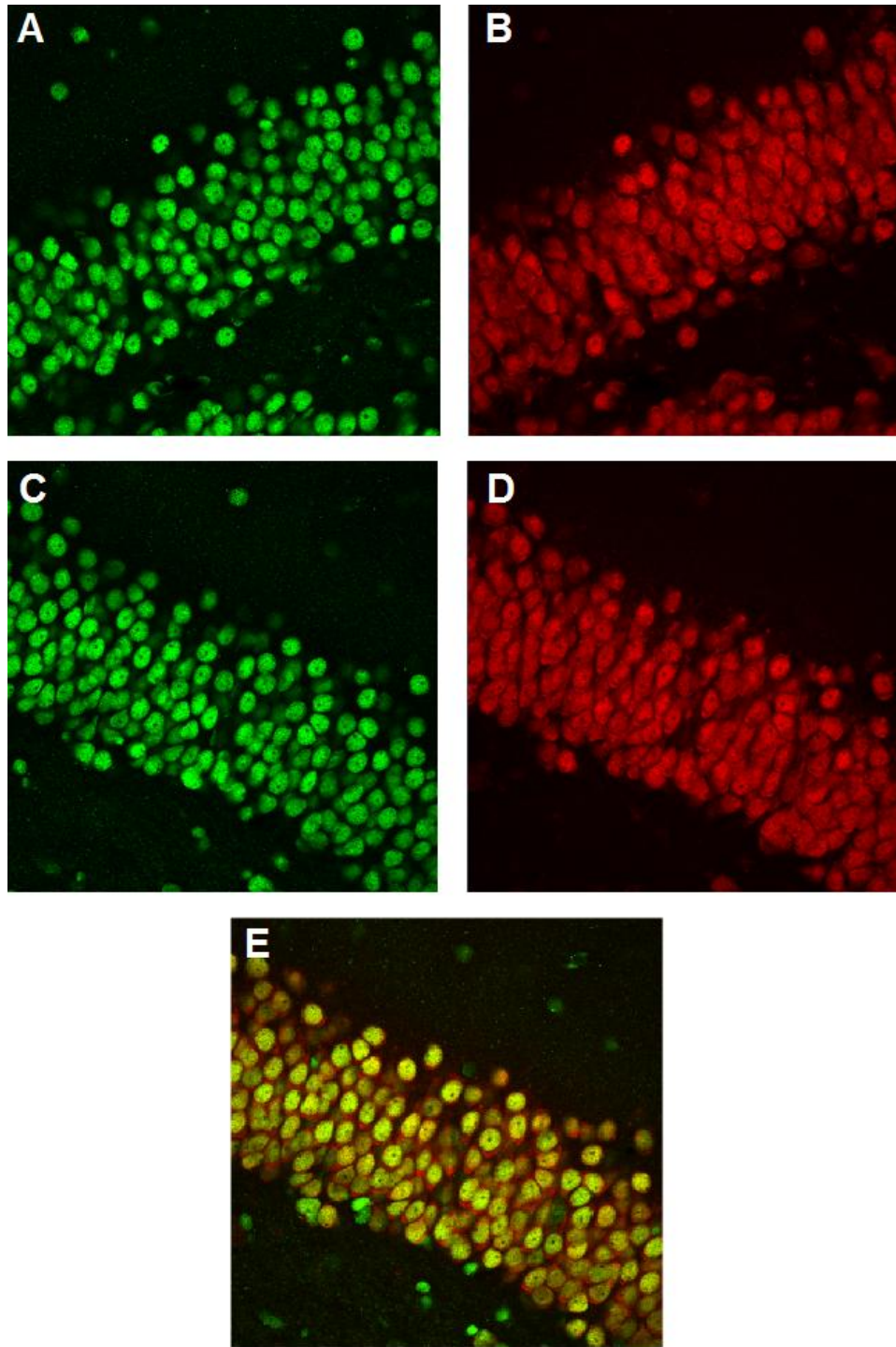


Figure 3.8. Photomicrographs of pCREB and NeuN immunoreactive cells in the GCL at 20x magnification. (A), (C) Cells positive for pCREB [green] in the GCL. (B), (D) The corresponding NeuN⁺ cells [red] in the GCL. (E) Merged image of pCREB and NeuN⁺ cells [yellow] in the GCL. Note that almost all GCL cells are neuronal in lineage (i.e., NeuN⁺).

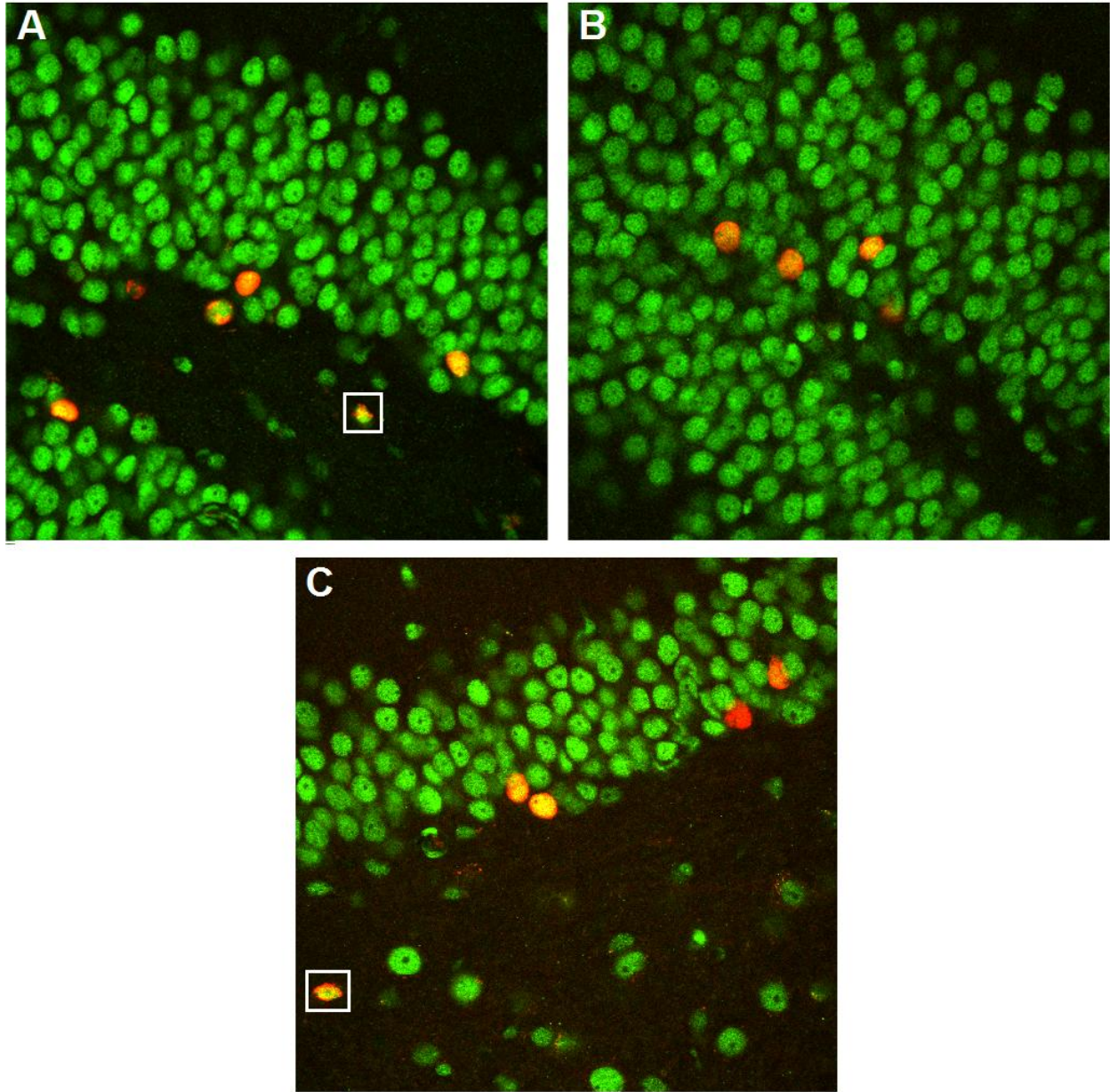


Figure 3.9. Photomicrographs of BrdU⁺ cells in the GCL at 20x magnification.

(A), (B), (C) Representative pictures illustrating BrdU⁺ cells [red] interspersed throughout the GCL, surrounded by BrdU⁻ cells [green]. Note that the boxed BrdU⁺ cells in Panel A and C would not be included in the final analysis due to their location (in the hilus) and morphological characteristics (too small and irregularly shaped, respectively).

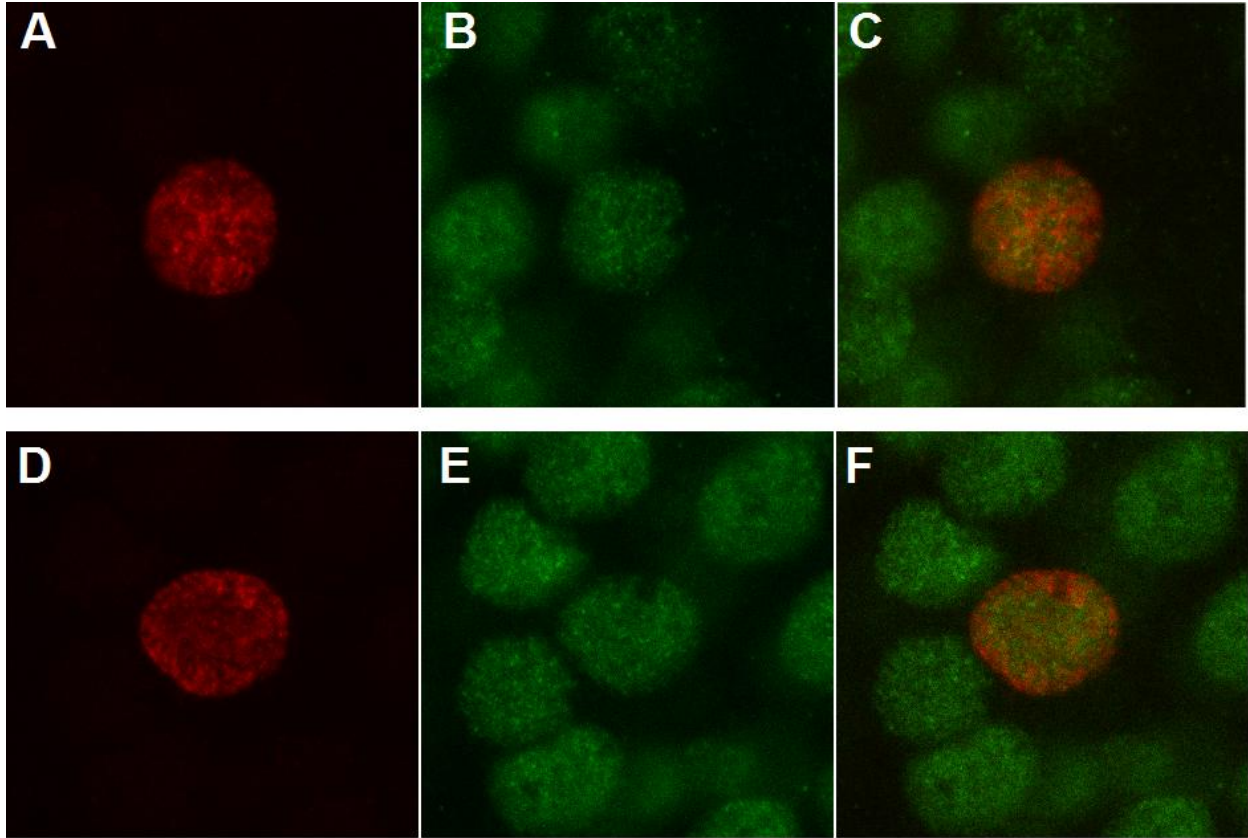


Figure 3.10. Photomicrographs of BrdU+pCREB double-labeled cells in the GCL at 63x magnification. (A) BrdU⁺ cell in the GCL. (B) pCREB⁺ cells in the region corresponding to panel A. (C) Panel A and B merged, clearly identifying which pCREB⁺ cell was recently generated. The BrdU+pCREB double-labeled cell in panel C would be quantified for pCREB expression using panel B. (D) BrdU⁺ cell in the GCL. (E) pCREB⁺ cells in the region corresponding to panel D. (F) Panel D and E merged, clearly identifying which pCREB⁺ cell was recently generated. The BrdU+pCREB double-labeled cell in panel F would be quantified for pCREB expression using panel E.

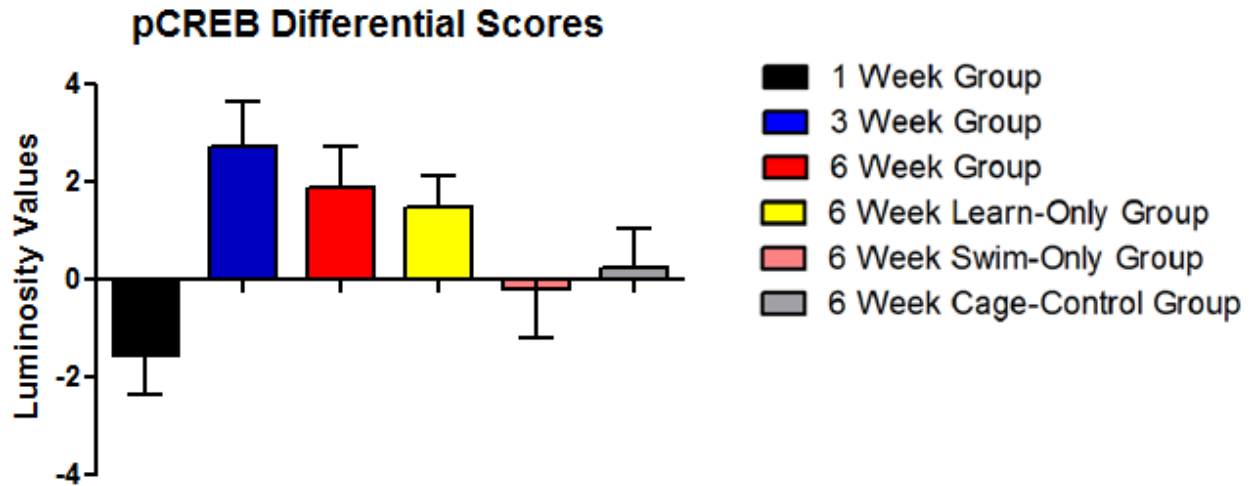


Figure 3.11. Comparison of the extent to which pCREB is differentially expressed in BrdU⁺ vs BrdU⁻ cells in response to the MWM task. pCREB differential scores were significantly greater in the 3 week vs 1 week group [$p = 0.018$] and neared significance in the 6 week vs 1 week group [$p = 0.077$]. The 0 on the Y-axis represents the average pCREB expression in BrdU⁻ cells. Error bars denote S.E.M.

Table 3.1. Mean pCREB expression scores across animals and groups. This table illustrates the mean pCREB immunoreactivity obtained from both BrdU⁺ and BrdU⁻ cells across each animal. By subtracting the scores obtained in BrdU⁻ cells from their BrdU⁺ counterparts, a pCREB differential score was calculated, with positive scores indicating higher pCREB activity in BrdU⁺ cells.

Rat ID	Group	pCREB in BrdU ⁺ cells	pCREB in BrdU ⁻ cells	pCREB Differential
1	1 week MWM	18.07	22.04	-3.98
2	1 week MWM	20.17	21.14	-0.98
3	1 week MWM	20.12	20.95	-0.83
4	1 week MWM	17.18	17.64	-0.46
5	3 week MWM	21.67	18.59	3.07
6	3 week MWM	20.83	18.13	2.70
7	3 week MWM	19.68	19.42	0.27
8	3 week MWM	20.39	15.56	4.83
9	6 week MWM	20.29	20.83	-0.55
10	6 week MWM	18.19	14.70	3.48
11	6 week MWM	18.83	16.74	2.09
12	6 week MWM	20.94	18.49	2.46
13	6 week learn-only	17.04	15.74	1.30
14	6 week learn-only	17.94	14.91	3.03
15	6 week learn-only	18.48	16.87	1.61
16	6 week learn-only	18.32	17.62	0.07
17	6 week swim-only	22.85	21.12	1.74
18	6 week swim-only	19.36	20.66	-1.30
19	6 week swim-only	18.72	19.80	-1.08
20	6 week cage-control	20.15	19.76	0.40
21	6 week cage-control	13.95	16.06	-2.11
22	6 week cage-control	16.25	15.11	1.14
23	6 week cage-control	16.08	14.60	1.48

the MWM as well as the single session rapid learning paradigm was well above baseline, but remained below levels seen in 3 week old neurons. Finally, pCREB expression in 6 week old neurons that were exposed to either cage-control or 'swim-only' conditions remained at around baseline levels (i.e., similar to that seen in average mature GCL neurons).

To determine whether pCREB expression was up- or down-regulated in a statistically significant manner across the groups, a one-way ANOVA was performed. This analysis found significance, [$F(5,17) = 3.59$, $p = 0.021$], with post-hoc Tukey tests indicating that this effect was driven by the difference in pCREB differential scores between the 1 and 3 week group [$p = 0.018$]. The difference in pCREB expression between the 1 and 6 week group was nearing significance [$p = 0.077$]. This result demonstrates that pCREB expression is differentially regulated in adult generated neurons in response to the learning and memory task.

Importantly, because learning and memory performance was consistent across groups, we can conclude that this effect is not due to any group simply displaying superior learning or memory. Instead, it follows that the effect is driven by intrinsic differences in the way pCREB is activated in immature hippocampal neurons of different ages and level of maturation in response to environmental stimuli.

If hyperphosphorylation of CREB plays a role in the preferential recruitment of 6 week old dentate granule neurons into learning and memory circuitry, one would expect pCREB to be more highly expressed in 6 week old BrdU⁺ neurons relative to their BrdU⁻ counterparts. In other words, one would expect the differential scores created in the current study to be in the positive (which they are) and significantly different from zero (with zero representing average pCREB expression in BrdU⁻ cells). To investigate this possibility, multiple one-sample t-tests were performed using the pCREB differential scores found in Table 4.1. As expected, pCREB expression was not significantly different in BrdU⁺ and BrdU⁻ cells in the cage-control [$t(3) = 0.280$, $p = 0.797$, $\eta^2 = 0.03$] or 'swim-only' conditions [$t(2) = -0.218$, $p = 0.848$, $\eta^2 = 0.02$]. Likewise, pCREB expression was not significantly different between BrdU⁺ and BrdU⁻ cells in 1 week MWM group [$t(3) = -1.921$, $p = .150$, $\eta^2 = 0.55$] or 3 week group [$t(3) = 2.894$, $p = .063$, $\eta^2 = 0.74$], although it was approaching significance in the latter case. Unfortunately, the difference in pCREB expression between BrdU⁺ and BrdU⁻ cells was also found to be non-significant in both the 6 week full MWM training [$t(3) = 2.178$, $p = .118$, $\eta^2 = 0.61$] and 6 week rapid learning

conditions [$t(3) = 2.471$, $p = 0.090$, $\eta^2 = 0.67$], although definite trends towards significance were found in both cases.

Although the t-tests failed to reach statistical significance, the effect size (η^2) scores in the 1, 3 and 6 weeks groups (i.e., the groups that were trained in the MWM) are large. In conjunction with the relatively small sample size ($n=3-4$) used in the immunohistochemical portion of this study, these high effect sizes suggest that the lack of statistical significance in these groups represent type II errors (false negatives). That is, while significant effects are present, the sample size (and thus power) is too small to detect it, statistically. How large of a sample would be required to achieve statistical significance in these tests? To answer this question, a post-hoc sample size calculation was conducted using *MorePower 6.0.*, a free statistical power calculator (Campbell and Thompson, 2002; Campbell and Thompson, 2012). For these analyses, the corresponding η^2 values for each group was used and power was held at the standard 0.8. The post-hoc sample size scores for the groups that underwent training on the MWM were calculated using one-way t-tests because the direction of their effects were demonstrated above (Figure 3.11). For the 6 week cage-control and swim-only groups, the power analysis indicated that 304 and 498 (respectively) more samples (i.e., animals or mean pCREB differential scores) would be required to achieve statistical significance. Given this result, confidence can be had that pCREB is not differentially regulated in 6 week old dentate granule neurons (relative to average neurons) in the cage-control and swim-only conditions. In dramatic contrast, the post-hoc power analysis indicated that only 2 more samples would need to be quantified in order to achieve statistical significance in the 6 week single-session learn-only group. Likewise, the power-analysis indicated that only 6 more samples would be required to achieve statistical significance in the 6 week full-MWM training group. In the 1 week MWM group, 8 more samples would be required to achieve statistical significance. And finally, the power analysis indicated that enough power was available to detect a difference in the 3 week group (which is essentially true, as $p = 0.062$ in this group). In sum, these post-hoc analyses are consistent with the idea that the current null results represent type II errors, and that the addition of a few more samples would likely result in statistical significance being achieved. The results of these post-hoc power analyses are enticing and consistent with a role of pCREB in the hyper-integration of immature neurons into learning and memory circuitry. It is for future studies to

determine whether statistically significant results materialize (e.g., in the 6 week MWM groups) when a larger sample size is implemented.

CHAPTER 4

DISCUSSION

4.1. Purpose of Current Study

The current work represents a first attempt at elucidating the molecular mechanism underlying the competitive advantage and accelerated integration of immature dentate granule neurons into spatial memory circuitry. Based primarily on its role in regulating intrinsic neuronal excitability and memory allocation, it was hypothesized that hyper-phosphorylation of the intracellular protein CREB in immature neurons might account, at least in part, for this preferential recruitment effect. To examine this possibility, a double label immunohistochemical approach was implemented in which one can visualize pCREB expression in adult generated hippocampal neurons of varying ages in response to a spatial learning and memory task.

4.2. Interpreting the Obtained Pattern of Results

Given that rates of integration into spatial memory circuitry progressively increase across 1, 3 and 6 week old dentate granule neurons (Kee et al., 2007a), the pattern of pCREB immunoreactivity found in the current study (i.e., Figure 43.11) might present itself as a bit puzzling. If CREB phosphorylation plays an important role in the incorporation of dentate granule neurons into learning and memory circuitry, one might expect to find a gradual increase in pCREB expression in dentate granule neurons as they mature from 1 to 6 weeks of age. In contrast to this expectation, the pattern of results obtained in the current study is as follows (outlined in Figure 3.11): One week old BrdU⁺ neurons express less pCREB than neighboring BrdU⁻ neurons. Three week old neurons express the greatest relative amount of pCREB immunoreactivity. Six week old dentate granule neurons display a high level of pCREB expression (relative to their BrdU⁻ counterparts) both in response to extended training on the MWM and in response to a single rapid learning session. However, the level of pCREB expression in these 6 week old neurons is still less than that seen in 3 week old BrdU⁺ neurons.

Six week old neurons exposed to a ‘swim-only’ condition do not display a significant change in pCREB expression, relative to neighboring BrdU⁻ neurons. And finally, 6 week old dentate granule neurons exposed to cage-control conditions are similar to neurons exposed to the ‘swim-only’ condition, in that their level of expression is unchanged from neighboring BrdU⁻ cells.

To make sense of the current pattern of results, I will be referring to and highlighting the work of Jagasia et al. (2009) which demonstrates that adult-generated neurons express different baseline levels of pCREB activation dependent upon their age and corresponding level of maturation. In particular, both 1 and 3 week old dentate granule neurons express relatively high (and approximately equal) basal levels of pCREB activity due to their ongoing morphological development. In contrast, 6 week old neurons express relatively low levels of pCREB activity under baseline, cage-control conditions. These results, in conjunction with the ‘preferential recruitment’ effect described by Kee et al. (2007a), readily explain the pattern of pCREB activity obtained in the current study.

4.2.1. pCREB Expression in One Week Old Dentate Granule Neurons

Given that one week old hippocampal neurons express a higher baseline level of pCREB immunoreactivity than their mature neuronal counterparts (Jagasia et al., 2009), why might these neurons express less pCREB than neighboring neurons in response to a learning and memory scenario? This result is explained by the fact that, in contrast to mature dentate granule neurons (i.e., the BrdU⁻ cells), hippocampal neurons generated one week earlier cannot yet integrate into surrounding neural circuitry in response to a learning and memory event (Kee et al., 2007a). Thus, that pCREB is expressed to a lesser extent in 1 week old BrdU⁺ neurons than their BrdU⁻ counterparts is consistent with a role of pCREB in regulating the integration of adult-generated neurons into hippocampal circuitry. This result also underscores the fact that the relatively high baseline level of pCREB activity found in 1 week old dentate granule neurons is still much lower than that seen in more mature neurons following a learning and memory scenario. In other words, the activity-dependent increase in pCREB expression that occurs in response to a learning and memory event by far exceeds the relatively high baseline level of pCREB activity found in immature dentate granule neurons.

4.2.2. pCREB Expression in Three Week Old Dentate Granule Neurons

Three week old adult generated hippocampal neurons have the same approximate probability of incorporating into spatial memory circuitry as fully mature dentate granule neurons. As such, the finding that 3 week old dentate granule neurons display greater pCREB immunoreactivity in response to spatial memory retrieval than both their BrdU⁻ counterparts and 6 week old dentate granule neurons seems at odds with the overarching hypothesis of the current study. Here, it is important to remember that 3 week old adult-generated hippocampal neurons express a high level of baseline pCREB activity due to their ongoing development (Jagasia et al., 2009). As such, the pCREB activity quantified in the 3 week old BrdU⁺ neurons in the current study is not a pure measure of activity-dependent expression in response to spatial memory retrieval. Instead, an additive effect of both developmentally-expressed pCREB and also the pCREB induced in response to the spatial memory trace occurs. That is, not only do these neurons express a high baseline level of pCREB due to their ongoing maturation, but their CREB is also phosphorylated in response to the MWM task. This additive effect is unique to the 3 week old hippocampal neurons in the current study and readily explains the high pCREB differential scores in this neural group.

4.2.3. pCREB Expression in Six Week Old Dentate Granule Neurons

Virtually all dentate granule neurons express some basal level of pCREB activation. However, unlike both 1 and 3 week old dentate granule neurons, 6 week old neurons do not express pCREB at levels beyond this relatively low level of baseline activity (Jagasia et al., 2009; Nakagawa et al., 2002ab). In line with this, pCREB expression in 6 week old cage-control neurons did not exceed the baseline set by mature dentate granule neurons in the current experiment. This result serves as an important control and indicates that the pCREB expression visualized in the current experiment is reliable and consistent with what has been published by other laboratories.

The animals injected with BrdU 6 weeks prior to training on the MWM are the most direct and important means through which the role of pCREB in the preferential recruitment effect was investigated in the current experiment. If CREB phosphorylation plays a role in regulating the accelerated recruitment of 6 week old dentate granule neurons into spatial memory circuitry, one would expect to see more intense pCREB immunoreactivity in 6 week old BrdU⁺

cells relative to their BrdU⁻ counterparts in animals exposed to the MWM task. Consistent with this, the overall pCREB differential score for 6 week old dentate granule neurons in response to the multi-day MWM protocol was in the positive direction. That is, 6 week old BrdU⁺ neurons preferentially expressed pCREB in response to spatial memory retrieval. This result would not be expected if pCREB did not play a role in the hyper-integration of 6 week old dentate granule neurons into spatial memory circuitry. Indeed, this apparent hyper-phosphorylation effect in response to the MWM probe test is consistent with the possibility that pCREB plays an important role in regulating the accelerated integration of immature hippocampal neurons into spatial memory circuitry.

Previous studies have shown that CREB plays a role in regulating memory allocation by biasing which neurons participate in the initial learning of a fear memory trace (Han et al., 2007; 2009). As such, CREB might be expected to play a role in regulating where information is stored in a neural circuit during the initial acquisition of a spatial memory trace. Importantly, sacrificing animals following a multi-day training protocol and probe test (i.e., the typical protocol used in MWM testing) does not readily capture the initial learning of a spatial trace. To better address this issue, the current experiment had a cohort of animals receive BrdU injections 6 week prior to engaging in a single session rapid learning protocol on the MWM. Relative to a training regimen that occurs over multiple days, this protocol readily isolates the initial acquisition period of spatial memory formation. Consistent with a role of CREB in biasing which neurons participate in the initial learning of a spatial trace, we found greater pCREB expression in 6 week old dentate granule neurons (relative to their mature BrdU⁻ counterparts) in response to the rapid, single session spatial learning protocol. Interestingly, pCREB levels in this subset of neurons are almost as high as is found in 6 week BrdU⁺ neurons that received a full five days of training in addition to a probe test. If pCREB truly plays a role in memory allocation in the dentate gyrus, then this result makes sense because the greatest competition for involvement and incorporation into a memory trace would likely occur during the initial formation of the circuit. Once the memory trace has stabilized (i.e., consolidated), the competition for incorporation into the trace would likely become less aggressive. Consistent with this idea, Reijmers and colleagues have shown that individual amygdala neurons that are involved in the initial acquisition of a (fear) memory trace are also involved with reactivation of this memory 3 days later (Reijmers et al., 2007). In other words, the neurons that won the competition for involvement into the circuitry

underlying the initial acquisition of the fear memory were the same ones activated in response to memory retrieval at a later time point. Consistent with this, the subset of adult-generated neurons that won the competition for involvement into the circuitry underlying spatial learning in the current experiment might be the same subset of neurons that become activated in response to the probe test a few days later.

Kee et al. (2007a) only considered whether immature hippocampal neurons are preferentially recruited into spatial memory circuitry after 3-5 days of training on the MWM. It is important to determine whether this effect is exclusive to retrieval of well formed memories or whether immature neurons also display hyper-integration in response to the initial formation of a spatial trace. If pCREB plays a role mediating the preferential recruitment effect, then the discovery of enhanced pCREB expression in 6 week dentate granule neurons in response to the single session rapid learning paradigm suggests that 6 week old dentate granule neurons might also be preferentially recruited into the circuitry underlying the initial acquisition of a spatial trace. In other words, the current results suggest that the accelerated integration of immature neurons into hippocampal circuitry probably also occurs in response to the initial formation of hippocampal-dependent memories. If this prediction turns out to be true, it would represent an interesting and important development in terms of the functional role played by adult generated hippocampal neurons. This possibility awaits experimental confirmation.

Exercise has positive effects on hippocampal physiology and function (e.g. van Praag et al., 2005). As such, it is possible that the increased pCREB immunoreactivity discovered in 6 week old dentate granule neurons exposed to MWM is due more so to the exercise involved with the task rather than to spatial learning and memory per se. To control for this possibility, a cohort of animals injected with BrdU 6 weeks earlier were placed in the water maze and allowed to engage in swimming exercise. Importantly, this swimming exercise had a negligible effect on the expression of pCREB in BrdU⁺ dentate granule neurons in these animals. Indeed, the relative pCREB expression in these 6 week old hippocampal neurons was no greater than that seen in BrdU⁺ cells of cage-control animals. This result provides evidence that the accelerated pCREB expression found in 6 week old dentate granule neurons in response to the MWM is not due to non-specific aspects of the task, such as swimming exercise.

4.3. Role of pCREB in Regulating the Preferential Recruitment of Immature Neurons into Spatial Memory Circuitry

The current work represents the first study to directly investigate the potential intracellular mechanism underlying the accelerated functional integration of immature hippocampal neurons into learning and memory circuitry. Three week old dentate granule neurons displayed greater pCREB expression in response to the MWM than did their one week old counterparts. Qualitative analysis of the obtained pattern of results is consistent with a role of CREB phosphorylation in the hyper-integration of immature (6 week old) dentate granule neurons into learning and memory circuitry. In an attempt to provide further evidence in favor of this possibility, multiple one-sample t-tests were performed using the pCREB differential scores found in Table 4.1. Unfortunately, the difference between pCREB expression in 6 week old BrdU⁺ neurons vs their mature neural counterparts in response to the MWM (i.e., the differential score) failed to reach statistical significance. Based on the small sample size used in the histological portion of this study in conjunction with large effect size discovered, this failure to obtain statistical significance likely represents a type II error. That is, a significant effect is likely present, but the current study lacked the power to detect it, statistically. Thus, the current results are perhaps best conceptualized as preliminary evidence in favor of a role for pCREB in the hyper-integration of immature dentate granule neurons into spatial learning and memory circuitry. However, future experimentation is clearly needed before definitive statements are made on this subject.

4.4. Future Research

Clearly, the first experiment that follows from the current thesis is replicatory with the addition of a larger sample size in the histological portion of the study. Beyond this replication, there are three important questions that should be addressed using tissue from the current study. First, do immature neurons that successfully integrate into spatial memory circuitry preferentially express pCREB? This question is a logical extension of the results obtained in the current work. To identify which neurons incorporated into the learning and memory circuitry underlying the MWM, the immediate early gene Arc (activity-regulated cytoskeletal-associated protein, or Arg 3.1) could be used. Arc protein has been linked to synaptic plasticity and memory, and is induced by spatial exploration in the same proportion of cells as is seen during electrophysiological

recordings (Gusowski et al., 1999; Vazdarjanova et al., 2002; Ramirez-Amaya et al., 2006). Thus, changes in the immediate early gene Arc correlate strongly with neuronal firing (Kee et al., 2007a). As such, Arc expression is often used as a marker of neural activity in response to spatial behaviours. Importantly, Arc expression is limited to neurons (NeuN⁺ cells) following spatial learning and memory tasks (Kee et al., 2007a; Ramirez-Amaya et al., 2006; Vazdarjanova et al., 2002). Thus, by examining the pattern of Arc expression in BrdU⁺ neurons after MWM performance, one can effectively identify which adult-generated neurons participated in the behavioural experience (Kee et al., 2007ab; Ramirez-Amaya et al., 2006; Ramirez-Amayas et al., 2007). Thus, a comparison between BrdU+Arc neurons and Arc-alone (i.e., Arc-BrdU⁻) neurons would allow for a comparison between the proportion of immature vs mature neurons that become incorporated into a spatial learning/memory trace. Given CREB's role in regulating intrinsic neuronal excitability and its purported role in memory allocation, one reasonable hypothesis is that 6 week old dentate granule neurons that successfully incorporate into the spatial memory trace (i.e., BrdU+Arc cells) would preferentially express pCREB. This experiment could also consider the different poles of the hippocampus, with the overarching hypothesis that the preferential recruitment effect and the hyperphosphorylation of CREB would be greatest in the dorsal hippocampus (Fanselow and Dong, 2010).

Second, the degree to which the preferential recruitment effect is specific to well-formed spatial memories should be addressed. In particular, it should be determined whether immature dentate granule neurons are preferentially recruited into the circuitry underlying the initial learning of a spatial trace. Given the pattern of pCREB immunoreactivity obtained in the 6 week 'learn-only' group in the current experiment, one might expect the preferential recruitment of immature neurons to occur in response to the initial acquisition of a spatial trace as well. Using an immunohistochemical strategy in which one can visualize the recruitment of adult-generated neurons into learning and memory circuitry (BrdU+Arc) (Kee et al. 2007b) in conjunction with the 'learn-only' animals of the current study, this question could easily be addressed. The role of pCREB in this effect could also be investigated (BrdU+Arc+pCREB). This experiment would represent an important step forward in terms of identifying the intracellular mechanisms and boundary conditions surrounding the preferential recruitment effect.

The final question that should be addressed using tissue from the current study relates to the closure of the critical period of competitive advantage and preferential recruitment. In

particular, do 10 week old dentate granule neurons maintain a phenotype conducive to hyper-integration into spatial memory circuitry? Using the immunohistochemical strategy set out by Kee et al. (2007b) in conjunction with the neural tissue of the 10 week group rats from the current study, this question could easily be addressed. Determining rates of pCREB expression in this group of animals would prove informative and interesting. For example, if the 10 week old neurons no longer possess a phenotype conducive to a competitive advantage in terms of memory allocation, then one would not expect these neurons to overexpress pCREB. This result would help explicate whether CREB phosphorylation plays a role in mediating the functional integration of immature dentate granule neurons. Further, the results of this experiment would be an important step in identifying the boundary conditions surrounding the preferential recruitment effect.

It is important to determine whether the preferential recruitment effect is specific to spatial learning and memory, or whether it translates into other hippocampal-dependent tasks. For example, following the protocol laid out in the current study, one could easily examine the role of adult-generated neurons of different ages in contextual fear conditioning or object recognition tasks. It is possible that the accelerated integration of immature neurons into hippocampal circuitry is specific to spatially-oriented tasks. However, a more parsimonious hypothesis is that this effect occurs in response to many hippocampal-dependent tasks, including fear conditioning and object recognition. The result of this experiment help delineate the functional role of neurogenesis in cognition. Furthermore, it is important to know the types of tasks onto which the preferential recruitment effect generalizes if we are to successfully manipulate neurogenic processes for clinical gain.

As discussed above (Section 1.7), evidence suggests that CREB regulates neuronal excitability by influencing the properties and distribution of ion channels on the cell membrane. However, given the current results, an important question remains: what underlies CREBs higher-than-normal propensity towards phosphorylation in response to spatial learning and memory in immature neurons? In other words, what processes lie upstream of CREB that account for its hyper-phosphorylation status in 4-8 week old dentate granule neurons? CREB is an intracellular protein, and as such there must be a signal from outside of the cell that triggers its activation. The initial over-expression of NKCC1 (a Cl^- importer) on the cell membrane of immature neurons leads to a high Cl^- content within the cell and represents an intriguing

mechanism upstream of CREB that might help account for its hyper-phosphorylation in immature hippocampal neurons. As was discussed above (section 1.5), GABA serves as an excitatory neurotransmitter to newly generated neurons (due to the overexpression of NKCC1). In fact, this property of GABA is critical for the proper dendritic and synaptic integration of newly generated neurons (Ge et al., 2006). Indeed, CREB phosphorylation in 1-3 week old developing adult generated neurons is in fact regulated by GABA-mediated depolarization (Jagasia et al., 2009). Given this, one possibility is that the accelerated functional integration of 4-8 week old hippocampal neurons is due to the initially excitatory effect of GABA in immature neurons. Unfortunately, the timeline during which GABA has an excitatory effect is too transient to account for the preferential recruitment effect, as this neurotransmitter has an inhibitory effect once the neuron reaches approximately 2-3 weeks of age (Ge et al., 2006). Thus, while GABA is certainly an important player in the proper development of newly generated neurons, it does not afford an adequate account of the critical 4-8 week period of accelerated functional integration seen in immature hippocampal neurons.

Experimentation into the role that NMDA plays in neurogenic processes has also been fruitful. For example, survival of adult generated neurons (up to 3 weeks of age) is competitively regulated by activation of their own NMDA receptors (Tashiro et al., 2006). Furthermore, NR2B-containing NMDA receptors are critical in mediating both the enhanced amplitude and decreased induction threshold for LTP experienced by 4-6 week old dentate granule neurons in vitro (Ge et al., 2007). Might NMDA receptor activity in immature neurons be responsible for the hyperphosphorylation of 4-8 week old dentate granule neurons? First, it is worth noting that the neurons used by Ge and colleagues no longer displayed hyper-plastic characteristics by the time they were 7 weeks old, and thus the timeline between the preferential recruitment effect and the NMDA-based effect is not fully harmonious. Furthermore, results regarding neuronal excitability and synapse formation in vitro do not necessarily translate into learning and memory in vivo. Regardless, the possibility that NR2B NMDA receptors play an important role in regulating CREB phosphorylation and thus in mediating the preferential recruitment effect is enticing. Evidence in favor of this possibility include the fact that CREB phosphorylation in hippocampal CA1 and DG neurons depends on intracellular Ca^{2+} influx through post-synaptic NMDA receptors (Porte et al., 2007). Furthermore, pulses of NMDA result in sustained pCREB activation for up to 3 hours in cultured neurons (Lee et al., 2005). And finally, neurons with

artificially induced increases in CREB activity show enhanced NMDA receptor-mediated synaptic responses (Marie et al., 2005). Together, these results emphasize the possibility that NR2B NMDA receptor activity underlies the activity-dependent increase in CREB phosphorylation found in immature neurons in the current study. This avenue of research warrants future investigation.

4.5. Potential Therapeutic Relevance

Adult hippocampal neurogenesis represents a candidate target for age-related cognitive decline (Drapeau and Abrous, 2008), epilepsy and seizure-induced cognitive impairment (Jessberger et al., 2007), and mood disorders such as depression (Duman, 2004; Sahay and Hen, 2008). Importantly, these disorders are associated not only with abnormal rates of neural proliferation, but also with abnormalities in functional integration into surrounding neural circuitry. For example, work from our lab has shown that seizure-generated neurons readily integrate into aberrant circuitry underlying seizure activity itself, but do not integrate into adaptive fear learning and memory circuitry (Fournier et al., 2011). As such, determining the molecular mechanisms that mediate the intrinsic excitability and integration of neurons into functional circuits could lead to the alleviation of both seizure activity and the cognitive dysfunctions that coincide with epilepsy (Botterill et al., 2011). Consistent with this possibility, mice developed with genetically lower levels of CREB are more resistant to pentylenetetrazol, kainite (Jancic et al., 2009) and kindling-induced seizures (Kojima et al., 2008). In sum, better delineating the intracellular mechanisms (e.g., CREB) that underlie the integration of adult-generated neurons might prove to be therapeutically relevant and therefore important.

CHAPTER 5

CONCLUSION

The purpose of the current thesis was to investigate the potential role of CREB phosphorylation in mediating the preferential recruitment of 6 week old dentate granule neurons into spatial memory circuitry. Although the overall trend of the data is consistent with this possibility, this effect failed to reach statistical significance. Based on the small sample size used

in the histological portion of this study in conjunction with the large effect sizes discovered, the failure to obtain statistical significance likely represents a type II error. That is, a significant effect is present, but the current study lacked the power to detect it statistically. Given this, the current results might be best conceptualized as preliminary support for a role of CREB phosphorylation in regulating the competitive advantage in memory allocation experienced by 6 week old dentate granule neurons. Determining the mechanisms that underlie the recruitment of adult-generated neurons into functional circuitry could prove important both theoretically and therapeutically. The role that CREB phosphorylation plays in this process warrants further investigation.

CHAPTER 6

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